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A STUDY OF THE DIGESTION OF PROTEIN IN HUMANS USING ILEAL AND FAECAL ASSAYS.

A thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Biochemistry at Massey University.

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*This thesis is dedicated to my husband,
Grant, and to my parents for always wanting
and expecting the best for me.*

ABSTRACT.

A study was conducted with 12 adult human subjects including six ilecstomates, to determine the digestibility of protein in a single mixed diet. Significant (P<0.05) differences were found between ileal and faecal amino acid digestibility values for most amino acids (histidine, arginine, aspartate, threonine, serine, glycine, proline, valine, leucine, phenylalanine, methionine, cysteine and tryptophan). The ileal digestibility coefficients ranged from 71.5 to 93.6% for glycine and lysine, respectively, whereas the faecal values ranged from 77.9 to 94.7% for glycine and leucine, respectively. The absolute differences between the methods ranged from 0.2 to 15.0% units for alanine/isoleucine and glycine respectively, and the average of the differences was 3.7% units.

The ileostomised growing pig (25kg) was investigated as a model animal to allow more routine determination of the ileal digestibility of protein in human foods, and good agreement was found between the species for apparent ileal amino acid digestibility. There were no significant differences between the two species for the apparent ileal amino acid digestibilities of amino acids, except for lysine, glutamate, proline and alanine.

The endogenous flows of amino acids at the terminal ileum were determined in both species, following consumption of a single protein-free meal. The amino acid compositions of the protein flows were similar for pigs and humans, with significant differences only being found for histidine, threonine, alanine, valine and methionine. The endogenous flows were used to correct apparent coefficients to give true estimates of digestibility. The latter values indicated near complete absorption of the dietary amino acids for the human subjects and growing pigs. When the interspecies comparison was based on the true digestibility values, there were only significant differences for the amino acids glutamate, phenylalanine, cysteine and methionine. The absolute differences

between the mean amino acid digestibility values for each species were smaller for true coefficients than for the apparent values.

The daily excretions of deoxyribonucleic acid (DNA) and diaminopimelic acid (DAP) were determined to indicate the levels of bacteria present at the terminal ileum and in the faeces of pigs and humans. There were higher levels of both of these marker compounds in human faeces samples than in ileal digesta. The opposite was observed for DNA in the pig, while the levels of DAP were similar at the two sites. The digestibility of fibre was also determined to indicate the extent of bacterial activity at these sites, and the values were greater in the faeces than in the ileostomy output of both species.

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TABLE OF CONTENTS.

			PAGE
Abstract			iii
Acknowledgements			v
Table of contents			vii
List of Tables			xii
List of Illustrations.			xvii
General Introduction			1
Chapter 1		Review.	7
1.1		Introduction.	7
1.2		The digestion of protein and absorption of amino acids.	8
	1.2.1	Gastric digestion.	8
	1.2.2	Small intestine.	9
	1.2.3	Large intestine.	13
	1.2.4	Factors affecting the digestion of dietary protein and amino acid absorption.	17
1.3		The *in vivo* determination of amino acid digestibility.	20
	1.3.1	Faecal versus ileal digestibility.	20
	1.3.2	Endogenous protein excretion from the small intestine.	22
	1.3.3	The use of non-digestible markers in digestion studies.	26
	1.3.4	The ileostomy model.	28
		a. Bacterial markers.	30
	1.3.5	Studies of protein digestibility using human subjects.	34
	1.3.6	The determination of amino acid versus nitrogen digestibility.	36

1.4	The role of model animals.	37
1.4.1	The stomach.	39
1.4.2	The small intestine.	43
1.4.3	The large intestine.	51
1.4.4	The gut microflora.	52
1.4.5	Comparative digestibility studies.	57
1.5	Overall conclusion.	59
Chapter 2.	Investigation of hydrolysis conditions for the determination of amino acids in biological samples.	60
Section 2.1	Acid hydrolysis for amino acid analysis.	63
Part 1	The use of sealed tubes versus round-bottomed flasks for acid hydrolysis of biological samples.	63
	a. Introduction.	63
	b. Methods and Materials.	65
	c. Results and Discussion.	68
Part 2	Investigation of hydrolysis time for the determination of amino acids in diverse samples.	71
	a. Introduction.	71
	b. Methods and Materials.	73
	c. Results and Discussion.	75
Section 2.2	Alkaline hydrolysis for the determination of tryptophan in biological samples.	81
	a. Introduction.	81
	b. Methods and Materials.	85
	Part 1. Comparison of sealed tubes with teflon containers for 4.3M LiOH hydrolysis of lysozyme and of a diet sample for the determination of tryptophan.	85

	Part 2. Investigation of the hydrolysis time required for maximum tryptophan values from a diet sample and ileal digesta and faeces samples pertaining to that diet.	87
	c. Results.	88
	d. Discussion.	92
2.3	Overall Conclusion.	95
Chapter 3	Celite as a non-digestible marker in digestibility studies with humans.	96
3.1	Introduction.	96
3.2	Animals, diets and procedures.	98
	a. Trial 1.	98
	b. Trial 2.	98
	c. Trial 3.	100
3.3	Chemical and statistical analysis.	101
3.4	Results and Discussion.	103
Chapter 4	The determination of apparent ileal and faecal amino acid digestibility values in adult humans receiving a mixed diet, and comparison with values determined with the growing pig.	108
4.1	Introduction.	108
4.2	Methods and Materials.	109
	a. Human study.	109
	b. Animal study.	115
4.3	Results.	121
4.4	Discussion.	127

Chapter 5	Determination of endogenous amino acids at the terminal ileum of the adult human and growing pig, and comparison between the two species of the true ileal digestibility of protein and amino acids in a mixed diet.	138
5.1	Introduction.	138
5.2	Methods and Materials.	140
5.3	Results.	143
5.4	Discussion.	146
Chapter 6	The use of bacterial markers to indicate the levels of bacteria present and the extent of bacterial activity in ileal digesta and faeces samples.	153
Section 6.1	The use of nucleic acids to indicate the levels of bacteria present in diet, ileal digesta and faeces samples.	155
	a. Introduction.	155
	b. Methods and Materials.	157
	c. Results.	160
	d. Discussion.	163
Section 6.2	The use of diaminopimelic acid to indicate the levels of bacteria present in diet, ileal digesta and faeces samples.	167
	a. Introduction.	167
	b. Methods and Materials.	168
	c. Results.	169
	d. Discussion.	171

Section 6.3	The determination of dietary fibre digestibility to indicate the extent of bacterial activity in the small and large intestines in humans and pigs.	173
	a. Introduction.	173
	b. Methods and Materials.	175
	c. Results.	181
	d. Discussion.	184
Section 6.4	Summary and Conclusion.	188
	Overall Summary and Conclusions.	190
Appendices.		193
References.		208

LIST OF TABLES.

TABLE		PAGE
1.1	The relative sizes and proportions of different parts of the digestive tract of the adult human (65kg) and the growing pig (25kg).	40
1.2	The secretion rate of some of the components of pancreatic juice in the adult human (65kg) and the growing pig (25kg).	45
1.3	The digestive enzymes present in the adult human (65kg) and the growing pig (25kg).	47
1.4	The pH of various regions of the digestive tract in the adult human (65kg) and the growing pig (25kg).	48
1.5	The secretion rate of some of the components of bile in the adult human (65kg) and the growing pig (25kg).	50
1.6	The main species of microorganisms ($\log_{10}$ viable count/g contents) found in various regions of the digestive tract of the adult human (65kg) and the growing pig (25kg).	55
2.1	Comparison of mean amino acid levels (nmol/mg freeze-dried sample) in a diet sample determined after hydrolysis in sealed tubes or round-bottomed flasks with or without prior oxidation with performic acid.	69
2.2	Slopes and intercepts for linear regressions of amino acid content on hydrolysis time for diet, ileal digesta and faeces samples.	76
2.3	Correction factors for the amino acids for which there was a curvilinear effect of time and showing a significant interaction (sample type*time) after hydrolysis of a diet, ileal digesta and faeces sample for 16, 24, 48 or 72h.	78

2.4	Correction factors for the amino acids for which there was a curvilinear affect of time and not showing a significant interaction (sample type* time) after hydrolysis of a diet, ileal digesta and faeces sample for 16, 24, 48 or 72h.	78
2.5	The recovery of 5-methyltryptophan (%), uncorrected and corrected tryptophan content (g/100g protein) in Lysozyme after 16, 20 and 24h hydrolysis in 4.3M LiOH in sealed tubes and teflon containers.	89
2.6	5-methyltryptophan recoveries (%) after 16, 20, 24 and 30h hydrolysis of a diet sample in 4.3M LiOH, and the uncorrected and corrected tryptophan values (nmol/mg freeze-dried sample) at these times using sealed hydrolysis tubes and teflon containers.	90
2.7	Corrected tryptophan determination (mg/100g freeze-dried sample) of diet, ileal digesta and faeces samples after 16, 18, 20, 24 or 30h.	91
3.1	Ingredient composition of a diet fed to young pigs to determine the recovery of Celite as acid-insoluble ash.	99
3.2	The recovery of added Celite ($X \pm S.E.$) in the faeces of young pigs (n=5; 12kg) and adult humans (n=6) or ileal output of pigs (n=6; 25kg) consuming diets consisting of ingredients commonly eaten by humans.	104
3.3	The apparent dry-matter digestibility ($X \pm S.E.$) for young pigs (n=5; 12kg) or adult humans (n=6) given a human-type diet, after total collection of faeces or by reference to a non-digestible marker.	105
4.1	Three alternative diets followed by 12 human subjects and that (diet C) given to 12 pigs.	110-111
4.2	Gross daily nutrient intakes of selected nutrients for the human subjects on 3 alternative diets.	112

4.3 Daily gross energy intakes of six ileostomates and six intact human subjects each on a constant diet for 7 days compared with their approximate daily energy expenditures. 114

4.4 Comparison of the chemical composition (mg/g dry-matter) and energy content (kJ/g dry-matter) of the diet followed by twelve human subjects and twelve pigs determined by reference to food table values or by chemical analysis. 122

4.5 Mean apparent ileal and faecal amino acid and nitrogen digestibility coefficients (%), for adult humans (65kg) and growing pigs (25kg) fed a meat/vegetable/cereal/dairy product based diet. 124

4.6 Levels of statistical significance for the differences between amino acid and nitrogen digestibility analysed within species or digestibility type. 125

4.7 Mean apparent ileal and faecal dry-matter digestibility coefficients (%) for adult humans (65kg) and growing pigs (25kg). 126

4.8 Mean apparent faecal gross energy digestibility coefficients (%) for adult humans (65kg) and growing pigs (25kg). 126

4.9 The differences for ileal and faecal amino acid and nitrogen digestibility coefficients between humans and pigs. 134

5.1 The ingredient composition of protein-free biscuits fed to ileostomised adult humans (65kg) and growing pigs (28kg) to determine the endogenous flows of amino acids at the terminal ileum. 141

5.2 Mean endogenous ilea flows of amino acids (mg/day) in adult humans (65kg) and growing pigs (28kg) following a single protein-free meal. 144

5.3 The mean true ileal amino acid and nitrogen digestibilities (%) for adult humans (65kg) and the growing pig (28kg) given a meat/vegetable/cereal/dairy products based diet. 145

5.4 The mean amino acid composition (%) of the endogenous protein at the terminal ileum of the 28kg pig and adult humans (65kg) after consumption of a single protein-free meal. 148

5.5 The differences between the adult human (65kg) and the growing pig (25kg) for the apparent and true ileal amino acid digestibility coefficients of a mixed diet. 150

6.1 The mean RNA concentrations (mg RNA-N:g Total N) in ileal digesta and faeces samples collected from adult humans (65kg) and growing pigs (25kg), and the mean daily output of RNA (mg RNA-N/day). 161

6.2 The mean DNA concentration (mg DNA-N:g Total N) in ileal digesta and faeces samples collected from adult humans (65kg) and growing pigs (25kg), and the mean daily output of DNA (mg DNA-N/day). 162

6.3 Mean diaminopimelic acid (DAP) concentration (mg/g dry-matter) in ileal digesta and faeces samples from adult humans (65kg) and growing pigs (25kg) consuming a meat/vegetable/cereal/dairy products based diet, and the mean daily DAP output (mg/day). 170

6.4 The sugar composition (mg/g dry-matter) of the dietary fibre fractions extracted from a meat/vegetable/cereal/dairy products based diet. 182

6.5 Mean apparent ileal and faecal digestibilities (%) of fibre components for adult humans (65kg) and growing pigs (25kg) fed a meat/vegetable/cereal/dairy products based diet. 183

6.6 Levels of significance for the differences in fibre component digestibilities between type (ileal and faecal) within species (human and pig) and between species within type. 183

LIST OF ILLUSTRATIONS.

FIGURE		PAGE
1.1	The prevalance (% of children in the community) of protein-energy malnutrition among children examined in surveys in over 60 countries.	2
1.2	a. The ileostomy stoma. b. The ileostomy stoma with everted end.	5
1.3	The Stickland reaction.	14
1.4	The digestive systems of adult humans and pigs.	38
2.1	A chromatogram of a mixture of amino acids including methionine sulphone, cysteic acid, diaminopimelic acid, tryptophan and 5-methyl-tryptophan.	67
4.1	The ileostomy operation performed on the 25kg liveweight pig.	between 115-116
6.1	A chromatogram of a mixture of alditol acetate derivatives of monosaccharides separated by gas-liquid chromatography.	180

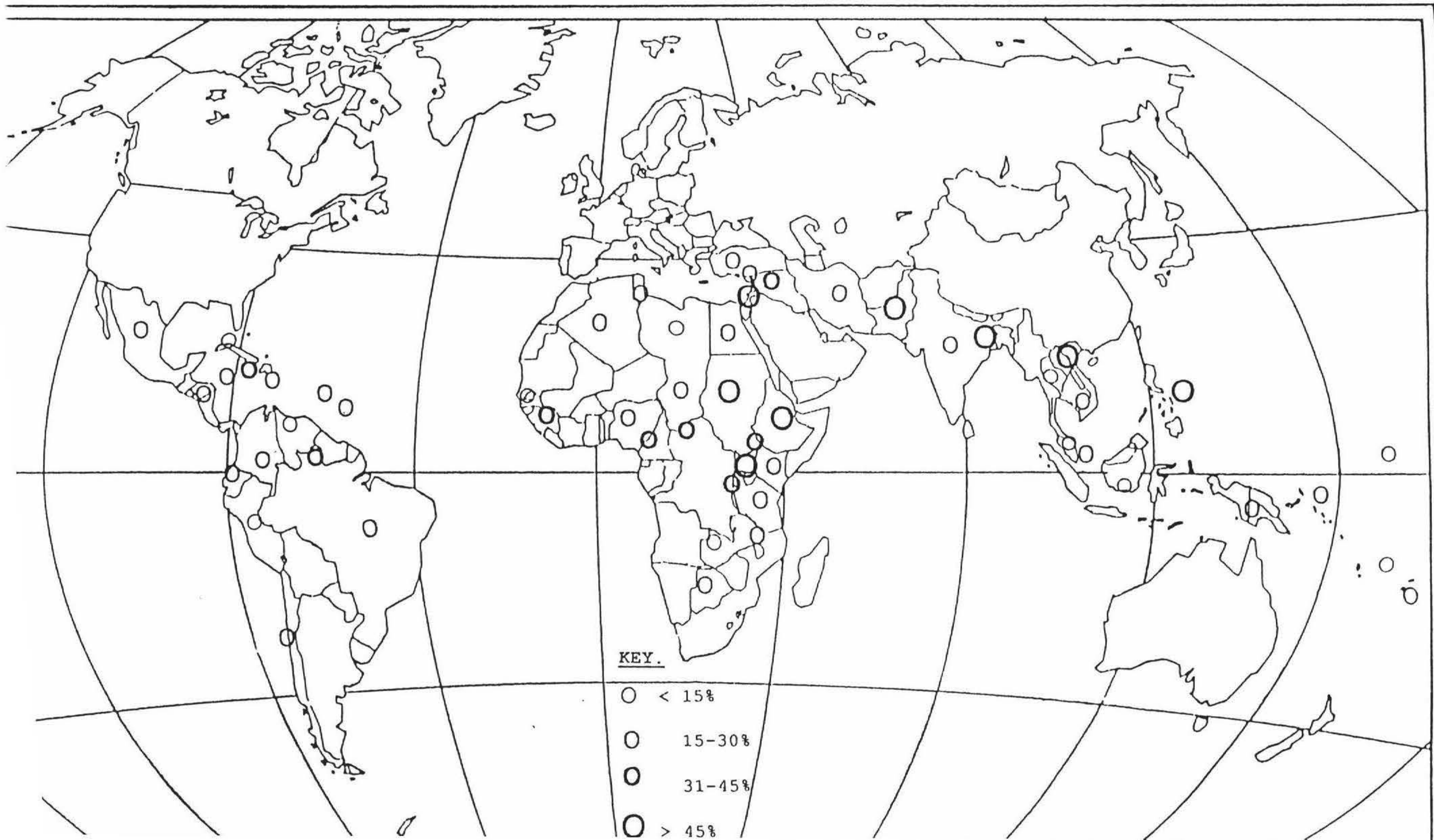
GENERAL INTRODUCTION.

The digestibility of a dietary component is a measure of its digestion and absorption in the gastrointestinal tract, whereas availability is the digestion, absorption and subsequent utilisation of the component. The measurement of the digestibility of a dietary component is important because it takes into account some of the biological processes occurring when food is ingested. The chemically determined composition of a diet alone, will not provide information on the digestibility of nutrients.

Dietary protein is a particularly important component of the human diet as it not only provides the essential amino acids required for protein synthesis and turnover in the body, which are isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, histidine and valine, but also energy, amino nitrogen (N) and sulphur. The determination of protein digestibility is important, therefore, and it is widely recognised that protein digestibility varies for different foodstuffs. If a deficiency of protein or any essential amino acid occurs over a prolonged period of time there will be detrimental effects on the health of the individual, particularly children, but also in adults. It is, therefore, important to be able to predict when such deficiencies may occur.

In most parts of the world, the diet of humans is made up of a mixture of protein sources which have different proportions of amino acids, and provides amino acids either in the required amounts or in excess. In many parts of the world, however, and particularly in underdeveloped countries, the levels of protein intake are very low and the protein quality is poor, often resulting in protein/energy malnutrition (PEM) (Fig.1.1), (Bengoa and Donosa, 1974). Some cultural limitations may be placed on the nature of protein sources given to children, and this in addition to a lack of education in the area of dietary protein quality can result in malnourished

Figure 1.1. The prevalence (% of children in the community) of protein-energy malnutrition among children examined in surveys in over 60 countries.
From: Bengoa and Donosa (1974).



children. Particularly in these countries, it is very important to have accurate values for the digestibility of protein and amino acids in foods, to allow assessment of the likelihood of protein deficiency occurring and the formulation of suitable diets.

More information on the quality of protein sources, from measurement of amino acid composition and digestibility should result in a more efficient and cost-effective use of the land, labour force and other resources, to produce and develop nutritionally beneficial foodstuffs, resulting in a healthier population, with lower medical cost to the country and lower mortality. Various diet preparations produced commercially from locally available sources have been developed for many underdeveloped countries, to provide nutritional but inexpensive foodstuffs (Orr, 1977). However, these types of preparations should be tested for the digestibility of amino acids and protein before being offered.

Moderate cases of PEM may also be found in the Western world in hospital patients and the chronically ill. These may be caused by poor nutrition or higher requirements due to illness, infection, healing after surgery, or a combination of these. Once again, a knowledge of the digestibility of amino acids in different foods will allow for more efficient diet formulation. Further, controlling the amount of ammonia produced from dietary protein in the large intestine, may be useful in the treatment of hepatic encephalopathy, and here knowledge of the ileal and faecal digestibilities of various diets would be required. Gibson _et al._ (1976) compared the amount of N excreted in ileostomy output and faeces for 2 levels of protein consumption to investigate the amount of protein entering the large intestine, however much more work could be done to look at a greater variety of foods and diets.

The digestibility of protein may be measured by either _in vivo_ or _in vitro_ methods. The _in vitro_ assays are simpler to carry out and often give more reproducible results, but it is difficult to simulate _in vivo_ digestion and absorption, and the results do not

necessarily reflect *in vivo* digestibility values (Dierick *et al.*, 1985; Graham *et al.*, 1985). For this reason the *in vivo* approach is usually preferred, when accurate estimates of digestibility are required. The traditional *in vivo* method in humans involves the quantitative collection of faeces (Istfan *et al.*, 1983; Schrimshaw *et al.*, 1983; Wayler *et al.*, 1983; Young *et al.*, 1984). The difference between the amount of dietary protein ingested and that remaining in the faeces is regarded as the amount of protein that has been digested and absorbed.

Based on work with simple-stomached domestic animals, however, it is now accepted both in theory (Payne *et al.*, 1968; Zebrowska, 1973c; Just, 1980; Low, 1980a; b; Rerat, 1981) and increasingly in practice (Low, 1982a; Jagger *et al.*, 1985; Moughan and Smith, 1985; Darcy *et al.*, 1985) that N, protein and amino acid digestibilities are more accurately determined by the ileal method. This involves measuring the amounts of N, protein and amino acids remaining in the digesta at the terminal ileum of the animal. The difference between dietary amino acid levels and those in the ileal digesta will indicate the extent of amino acid absorption that has occurred in the small intestine.

It has been noted (Bayley *et al.*, 1974; Holmes *et al.*, 1974; Braude *et al.*, 1975; Low, 1975) that the digestibility of N, protein and amino acids were on average 5% lower at the terminal ileum than when measurement was made on the faeces, and sometimes lower still, especially for poorly digested diets. As yet, no studies have been undertaken to investigate if a difference exists between the faecal and ileal methods for the determination of amino acid digestibility in humans. The first aim of the present study, therefore, was to investigate the digestibility of protein in humans by the ileal and faecal methods, and to determine if there were differences in amino acid digestibility when using these two methods. The collection of ileal digesta was made by using the ileostomate, who has had the terminal ileum exteriorised because of disease of the large intestine (Fig.1.2).

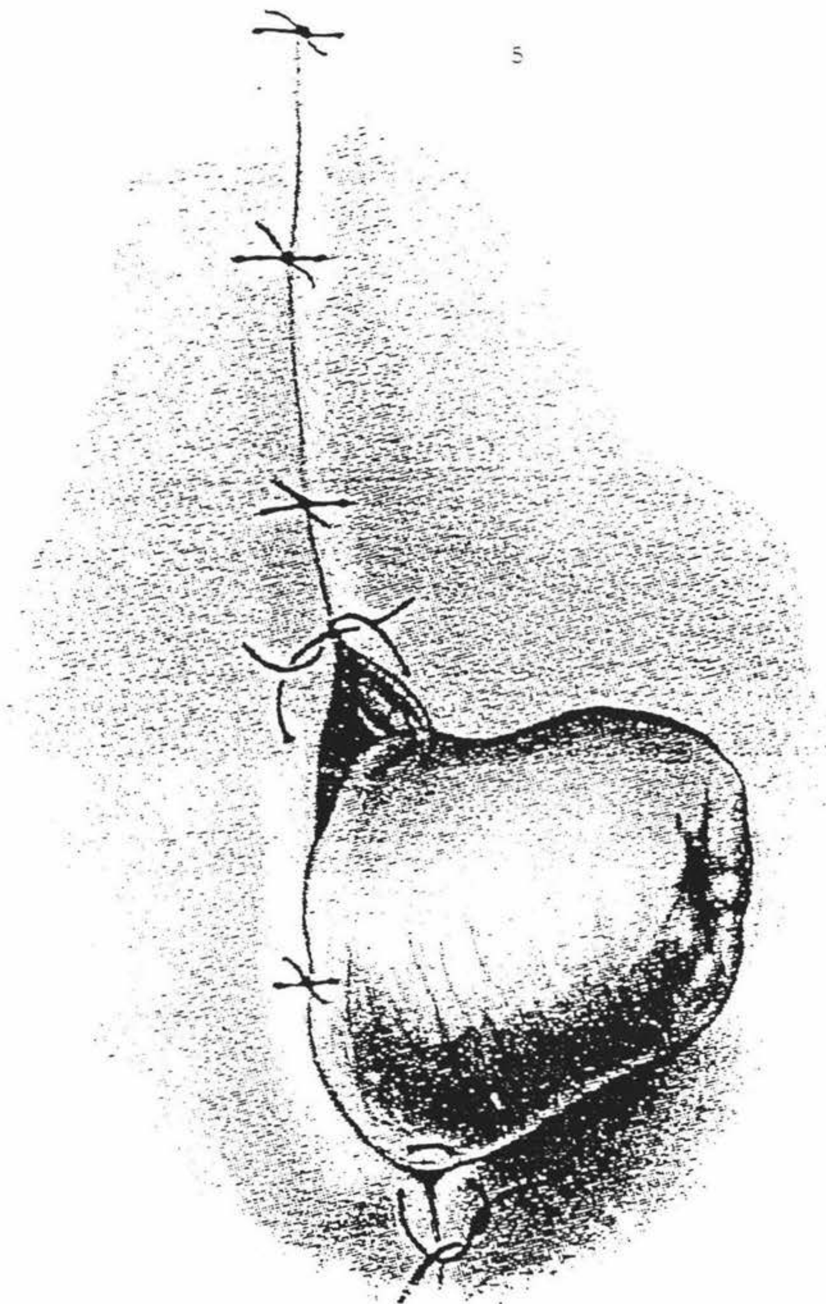
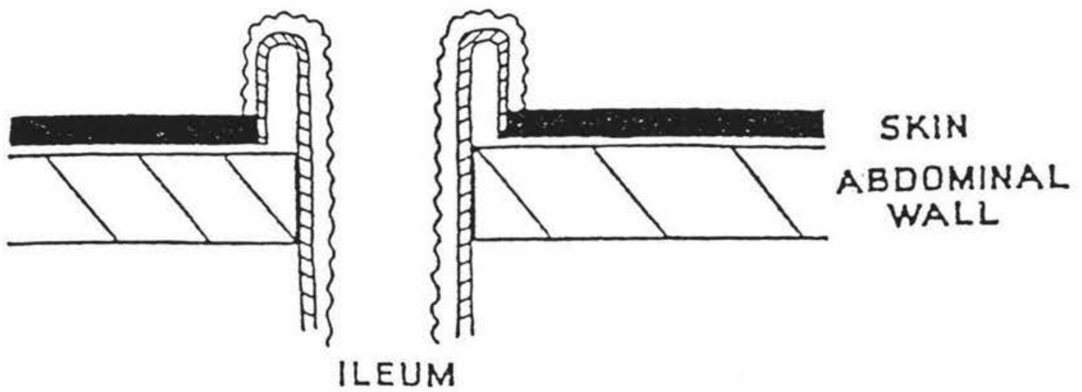


FIGURE 1.2.a. The ileostomy stoma. From: Brooke (1969).

FIGURE 1.2.b. The ileostomy with everted end. From: Brooke (1952).



Regardless of whether ileal or faecal amino acid digestibility was found to be the preferred method in humans, it would be desirable to use a model animal for the human to allow routine and more controlled studies to be undertaken. Because of the similarities in aspects of digestive physiology between the pig and the human, it appears that the pig may be a suitable model animal. A second aim of this study, therefore, was to compare ileal and faecal protein and amino acid digestibilities between pigs and humans, when they consumed the same diet.

CHAPTER 1.

REVIEW.

1.1. INTRODUCTION.

The processes of protein digestion and absorption are complex, and may be influenced by many factors. An understanding of these is important, therefore, when designing, conducting, and interpreting results of studies on protein digestibility. Also, there are different approaches available for determining digestibility *in vivo* and the most appropriate method must be chosen. The use of animal models in biomedical research, and the suitability of different animals for certain aspects of research, have been reviewed by Waddell and Desai (1981). In the area of nutrition there are three main choices of animals. The pig, the rat and primates, all of which have broadly similar digestive anatomy and physiology, have been used as models for human nutritional research. Nyman *et al.* (1986) have suggested that larger animals may be more suitable as model animals, as smaller animals consume more energy/unit body weight, with a faster transit of food through the gut, and therefore possibly lower nutrient utilisation. Primates are difficult and costly to obtain and maintain, and therefore the pig is a popular choice, and is one of the best models available for studies on the omnivorous human (Miller and Ullrey, 1987). However, a more detailed examination of the pig as a model animal for human digestibility studies is required.

The above topics will be reviewed in the following sections in the context of the overall aims of the present study.

1.2. THE DIGESTION OF DIETARY PROTEIN AND ABSORPTION OF AMINO ACIDS.

The processes of protein digestion and the absorption of amino acids occur throughout the digestive tract. The ingested food is partly broken up by the mechanical action of chewing by the teeth in the mouth. The food is then swallowed and enzymatic digestion of the protein begins in the stomach.

1.2.1. GASTRIC DIGESTION.

The gastric digestion of protein is carried out by pepsins (E.C. 3.4.23.1) secreted by the chief cells in the stomach. The precursors of pepsins, the pepsinogens, are activated by hydrochloric acid also secreted in the stomach, and by the autocatalytic activity of pepsin. There are two groups of pepsins which have distinct pH optima for protein digestion in the human gastric juice. The groups are separated on the basis of electrophoretic properties, and seven pepsinogens have been shown to exist in the human gastric mucosa (Bockus, 1976a). Group I, pepsins 1-5, have a pH optimum of 1.6-2.4 and occur only in the proximal stomach, while group II, pepsins 6 and 7, have a pH optimum of 3.3-4.0, and are found throughout the stomach and duodenum (Taylor, 1959). The two groups have other differences, but in general all pepsins act fairly non-specifically as endopeptidases particularly attacking the peptide linkages in which the amino groups of aromatic amino acids are involved.

Borgstrom *et al.* (1957), in a study using human subjects fed a radio-iodinated protein, have suggested that 10-15% of protein ingested may be digested in the stomach. That study has shown that only 85-90% of the ingested ^{131}I in digesta taken from the pylorus region was in a form able to be precipitated by phosphotungstic acid. An earlier study by Beazell (1941) also reported that up to 10% of ingested protein in human gastric digesta is soluble in tungstic acid. Studies using gastrectomised patients have given conflicting results as to the significance of gastric protein digestion, some showing that it was not essential and gastrectomy

had little effect on the overall N absorption in the gut (Taylor, 1968), while other studies reported a decrease in protein absorption of 10-25% in gastrectomised patients as compared to controls (Jeejeebhoy, 1964). Also, studies (Zebrowska, 1973c; Low, 1979b; Zebrowska et al., 1983) using the pig have shown that up to 20% of dietary protein may be partly digested in the stomach. Therefore it appears that digestion of protein in the stomach of monogastric animals may be quite considerable, and is probably important to overall digestion in the gut. There appears to be no absorption of amino acids in the stomach (Zebrowska, 1980; Zebrowska et al., 1983).

1.2.2. SMALL INTESTINE.

The digestion of dietary protein and peptides continues in the small intestine by enzymes secreted from the pancreas, and by intestinal enzymes that are either secreted into the lumen of the gut, attached to the brush border of the intestine, or are active within the mucosal cells. The pancreatic proteolytic enzymes are secreted as their inactive precursors (zymogens) and are activated in the gut. These enzymes are trypsin (E.C. 3.4.21.4), chymotrypsin (E.C. 3.4.21.1), carboxypeptidases A (E.C. 3.4.2.1) and B (E.C. 3.4.2.2), and elastase (E.C. 3.4.21.11). Trypsin is converted from its zymogen by the protease enterokinase, which is secreted by the duodenal mucosa. Trypsin then activates the other enzymes by proteolysis, and it is also autocatalytic. Trypsin, chymotrypsin and elastase are endopeptidases and the carboxypeptidases are exopeptidases. Trypsin hydrolyses the peptide bonds on the carboxyl side of lysine and arginine only, while chymotrypsin only cleaves the peptide bonds on the carboxyl side of the aromatic amino acids tyrosine, tryptophan and phenylalanine, as well as large hydrophobic residues such as methionine (Stryer, 1981). The substrate specificity of elastase is different again, only cleaving peptide bonds adjacent to uncharged amino acid residues, such as alanine, valine, leucine, isoleucine, glycine and serine (Stryer, 1981). Carboxypeptidase A cleaves aromatic and aliphatic amino acids from the carboxyl terminal of the

peptide chain most readily, and carboxypeptidase B prefers basic residues (Stryer, 1981). The intestinal enzymes (enteropeptidase, aminopeptidases, di-, tri- and tetrapeptidases) are formed in the mucosal cells. Enteropeptidase is secreted into the lumen of the gut, while the presence of other peptidases in the lumen of the gut is due to desquamated cells (Smith et al., 1983). Peptidases are integral components of the brush border of the intestine further hydrolysing peptides before absorption (Das and Radhakrishnan, 1979). The peptidases also act within the cell hydrolysing peptides to their free amino acids for absorption into the blood and lymph circulations.

The digestion of protein in the small intestine may also be carried out by enzymes synthesised by gut microorganisms, some of which have proteolytic activity. Studies using germ-free animals have shown that intestinal enzymes may be degraded in the gut by microorganisms, which may influence the digestion of protein, both of dietary and endogenous origin (Snook, 1973). However, there appears to be an apparent over-production of pancreatic enzymes, exceeding that required for hydrolysis of ingested protein (Zebrowska et al., 1983). The effect of microbial action in the small intestine is likely to be small as indicated by the low ammonia and branched chain volatile fatty acid concentrations in this part of the gut measured in the digesta taken from sudden death victims (Macfarlane et al., 1986). Another contributing factor to suggest that microbial protein digestion may be negligible in the small intestine is the low numbers of bacteria found here compared to the large intestine, and the rapid passage of digesta through this part of the gut (Clemens et al., 1975). Salter and Coates (1971) found that the microflora of the gut in chickens appeared to have no significant effect on the products of protein digestion in the upper tract, in a study comparing conventional and germ-free birds. In other studies (Coates et al., 1973; Salter and Fulford, 1974) using conventional and germ-free chickens, the gut microflora appeared to have little influence on the digestion of protein as there was no difference in apparent or true digestibilities for

virtually all amino acids for two diets, between the two types of birds.

The absorption of the products of enzymatic protein digestion, which are amino acids and peptides, occurs in the small intestine. Absorption of amino acids in the intestine is specific, active and possibly dependant on Na^+, although this has not yet been clearly established in humans (Davenport, 1982; Silk et al., 1985). The L-isomers of amino acids are absorbed faster than D-isomers. Kinetic studies (Elsden et al., 1950; Gibson and Wiseman, 1951) on the absorption of amino acids from the small intestine of experimental animals has led to the discovery of specific absorption mechanisms that exist for different groups of amino acids. There are specific mechanisms for the absorption of neutral amino acids, basic amino acids, dicarboxylic amino acids, and for N-methyl amino acids, respectively. The first three mechanisms definitely exist in humans (Davenport, 1982; Silk et al., 1985). There is not normally any competition between the mechanisms for absorption of amino acids, but there may be competition between amino acids within a group (Bockus, 1976b).

In addition to the absorption of free amino acids, absorption of peptides of 2-6 amino acids occurs in the small intestine (Agar et al., 1954; Newey and Smyth, 1959; Adibi, 1971; Hellier et al., 1972; Silk et al., 1985). Nixon and Mawer (1970a) investigated the release of amino acids during digestion and the rate of absorption of free amino acids in humans. They suggested that absorption of lysine, valine, arginine, tyrosine, phenylalanine, methionine and possibly leucine could be due to absorption of their free amino acids, but that the other amino acids must have been absorbed by other mechanisms, probably peptide absorption, because their rate of release during digestion was too slow to allow for the absorption rates observed for the free amino acids. The digestion of absorbed peptides continues within the mucosal cells with the free amino acids being released into the circulation.

Borgstrom et al. (1957) have shown that the absorption of amino acids and peptides begins in the duodenum and is 80% complete in the first 50-100 cm of jejunum in humans. Nixon and Mawer (1970b) have also shown this, but they used a highly digestible protein source, and the effect is likely to vary with source (Rerat, 1981). Adibi and Mercer (1973) demonstrated that some dietary amino acids remained in the digesta at the terminal ileum, especially when a poorly digested diet was fed.

Free amino acids are absorbed at a greater rate than peptides in the duodenum of experimental animals (Buraczewska, 1980; Zebrowska, 1980), but in the rest of the small intestine the rate of absorption of peptides is greater than for free amino acids, in humans (Adibi and Mercer, 1973; Bockus, 1976b). A study (Buraczewska, 1977, cited by Zebrowska, 1980) using pigs has shown that the rate of amino acid absorption is lower in the proximal small intestine than in the central and distal parts. Zebrowska et al. (1975) have shown that with pigs the most extensive N absorption occurred in the middle third of the small intestine.

Non-essential amino acids are generally less efficiently absorbed than essential amino acids in the small intestine (Buraczewska, 1980; Nolan, 1985). In the pig, methionine, leucine, isoleucine and arginine are the most rapidly absorbed amino acids in the whole small intestine, and histidine, threonine, and lysine the slowest (Zebrowska, 1973c). Different parts of the intestine have different abilities to absorb individual amino acids, for example, threonine, glycine and alanine are more rapidly absorbed in the proximal small intestine than in distal parts (Zebrowska, 1980; Rerat, 1981), phenylalanine, tyrosine and leucine are most rapidly absorbed in the distal parts, where the non-essential amino acids are the slowest absorbed (Low, 1976).

The concentration of amino acids in the mucosal cells builds up during digestion and absorption, and hydrolysis of absorbed peptides to the constituent free amino acids occurs in the cells (Bockus,

1976b; Davenport, 1982). Free amino acids are then absorbed into the blood and lymph circulations, making them available to the body for utilisation (Bolton and Wright, 1937).

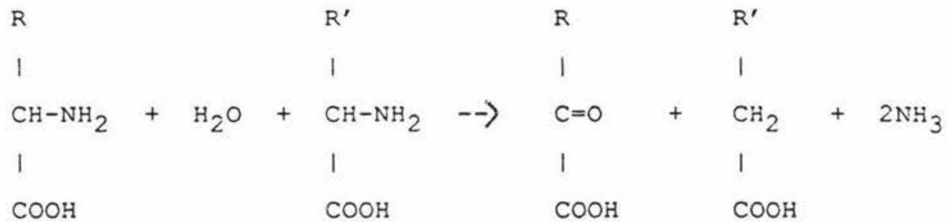
1.2.3. LARGE INTESTINE.

The digestion of protein is continued in the large intestine by the action of the microbial enzymes. This site is probably most significant for the digestion of endogenous protein entering from the small intestine (Snook, 1973; Wrong et al., 1981).In addition to the proteolytic activity demonstrated by the bacterial enzymes, the bacteria of the hindgut in experimental animals can modify nitrogenous compounds, by processes such as the deamination and decarboxylation of amino acids, degradation of Maillard compounds (amino acid-sugar complexes), mucosal residues, mucus glycoprotein, uric acid, urea, and amines (Snook, 1973; Drasar and Hill, 1974; Mason, 1980; 1984). All these activities are likely to also exist in humans (Bockus, 1976c). Michel (1966) has shown that all amino acids can be broken down in vitro, by caecal microorganisms, but at different rates.

The principle reaction catalysed by microbial enzymes involving primary amino groups is deamination with the production of ammonia (NH_3) (Drasar and Hill, 1974). There are several different pathways for the deamination of amino acids, the most widely reported route being the reduction of amino acids with the formation of a saturated fatty acid. There is also oxidative deamination with the formation of an aldehyde, although this reaction is restricted to a few microorganisms including E.coli, acting on a restricted range of amino acids (Drasar and Hill, 1974). There may also be reactions resulting in the production of an α-hydroxy acid, removal of elements of NH_3 with the formation of an unsaturated product, and the Stickland reaction (Fig.1.3), involving 2 amino acids, one being oxidised to its keto acid, and the other reduced (Drasar and Hill, 1974). The products of deamination include succinate and fumarate from aspartate, indole propionate and indole pyruvate from

tryptophan, and other phenolic substances from tyrosine and phenylalanine (Wrong et al., 1981).

Figure 1.3. The Stickland reaction.



These pathways are common to a large number of microorganisms particularly enterobacteria and clostridia found in the large intestine, and in ileostomy output (Drasar and Hill, 1974; Wrong et al., 1981). Microorganisms produce deaminases optimally in vivo, at alkaline pH, so the production of organic acids tends to lower the pH towards neutral (as is found in the small intestine) so limiting their activity (Drasar and Hill, 1974). Amines formed by the initial decarboxylation of amino acids are also metabolised by these pathways (Wrong et al., 1981). The metabolism of secondary amino groups is mainly by N-dealkylation and N-nitrosation (Drasar and Hill, 1974).

The amino acid decarboxylases have been described by Gale (1940; 1941), and each enzyme is specific for one amino acid. Decarboxylases have been described for arginine, lysine, histidine, tyrosine, glutamate, aspartate, tryptophan and ornithine. Hawksworth (1973, cited by Drasar and Hill, 1974) desribed another enzyme specific for proline, and Haughton and King (1961) described a further enzyme with wider specificity, that is active towards leucine, isoleucine, valine, nor-valine and α-amino butyric acid. Decarboxylases have higher activity in acidic conditions, with pH optima around 5.0 (Wrong et al., 1981) and so are likely to have low activity in the large intestine, and the lower small intestine, where the pH is around neutral. Some of the strains of bacteria found in the small and large intestines, such as E.coli and some

strains of clostridia and bacteroides, produce decarboxylases, while other strains that may be present, such as the streptococci, may not produce decarboxylases (Drasar and Hill, 1974).

Amino acids may also be metabolised by fission reactions producing cyclic compounds, other amino acids, α-keto acids and NH_3 (Mason, 1984). For example, E.coli and some clostridia can split tyrosine, streptococci can hydrolyse arginine, and tryptophan can be degraded to indole by some enterobacteria, some fusobacteria and some bacteroides (Drasar and Hill, 1974), all of which are found in the large intestine and to a lesser extent in the small intestine.

As well as degradation of nitrogenous compounds, the microflora of the large intestine can use the breakdown products for synthesis of their own proteins and other cellular components, and so changing the amino acid composition of the digesta. The main product used for this is NH_3, but peptides and amino acids may also be used directly (Payne, 1975). The amino acid composition of the faeces is surprisingly constant for different diets and conditions, and so probably reflects the composition of microbial protein (Kidder and Manners, 1978; Mason, 1980). Overall the large intestinal metabolism will result in a change in the relative proportions of amino acids, with a loss of most amino acids, but a gain in some such as methionine and lysine (Mason, 1980; Rerat, 1981).

There is in vitro evidence that amino acids may be actively absorbed into the mucosal cells of the large intestine of experimental animals. However there is little evidence that further absorption of amino acids occurs from the mucosal cells to other cells or into the circulation, (Wrong et al., 1981; Nolan, 1985). Indirect evidence from early studies using human subjects led workers to believe that nutritionally significant quantities of amino acids were absorbed from nutrient enemas in the large intestine (Wrong et al., 1981). This was based on observations of changes in urinary N, and the disappearance of protein determined by washing out the large intestine several hours after the enema. However, the larvage

methodology has since been criticised as being unphysiological (Wrong *et al.*, 1981). Fordtran *et al.* (1964) found that the tryptophan metabolites indican and indole-3-acetic acid rapidly increased in the urine after tryptophan was infused *per rectum* into normal subjects, suggesting that at least the carbon-skeleton of tryptophan is absorbed in the large intestine. Studies using experimental animals (Slade *et al.*, 1970; 1971; Hoover and Heitmann, 1975) have suggested that absorption of amino acids does occur in the large intestine because of improved N retention observed when urea was added to the diet of horses and rabbits. Other workers have demonstrated caecal breakdown of some amino acids only, with a small amount of direct absorption. For example Olsewski (1975, cited by Low, 1980a) used virtually sterile caecal pouches and reported that only very small amounts of amino acids were absorbed in pigs. Other studies, both *in vitro* and using experimental animals, have also demonstrated a limited degree of amino acid uptake in the large intestine, but the movement appeared to be by passive diffusion as active transport did not occur (Binder, 1970; Wrong *et al.*, 1981). Further, the concentrations of faecal free amino acids are lower than the plasma amino acid concentrations (Owens and Padovan, 1975), so it is unlikely that net absorption of amino acids could occur by passive diffusion (Wrong *et al.*, 1981).

A considerable amount of evidence exists, therefore, to suggest that protein or amino acids are not absorbed to any great extent in the large intestine. Further, several workers have demonstrated no improvement in N balance with infusion of protein and amino acids into the large intestine (Zebrowska, 1973c; Hodgson *et al.*, 1977; Gargallo and Zimmerman, 1981; Just *et al.*, 1981). Zebrowska (1973c) has shown that enzymatically hydrolysed casein infused into the terminal ileum of pigs on a protein-free diet (in negative N balance) was decomposed and the N absorbed in the large intestine, then rapidly and almost completely excreted in the urine. It therefore appears that while some limited absorption in the large intestine, may occur for some amino acids, it is generally considered that the amino acids and/or peptides entering the large

intestine are not available to the host in nutritionally significant quantities.

A large portion of the NH_3 formed in the hindgut may come from the microbial breakdown of urea (Nolan, 1985) which is secreted into the gut in saliva, gastric juice, bile and other secretions. Urea is the major end product of N metabolism in animals and humans, and the gut is possibly an important excretion route (Wrong et al., 1981). Urea is probably not absorbed from the large intestine but is broken down to NH_3 and carbon dioxide (Wrong et al., 1981, Nolan, 1985). Overall, it appears that most of the N absorbed in the large intestine is in the form of NH_3 (Rerat, 1981; Wrong et al., 1981). Amines, however, formed by microbial action may also be absorbed in the large intestine (Mason, 1980).

1.2.4. FACTORS AFFECTING THE DIGESTION OF DIETARY PROTEIN AND AMINO ACID ABSORPTION.

Many factors, both external and internal, can affect the processes of protein digestion and absorption of amino acids throughout the digestive tract. Some external factors that may affect these processes include the age, sex, race, physiological status or health and fitness of the subject, as well as the use of medication, alcohol consumption, and dietary habits. Low levels of nutrient intake over extended periods of time may improve the absorptive capacity of the intestine. Semistarvation in rats improved histidine and glucose absorption (Kershaw et al., 1960). Also histochemical observations after dietary stress have shown that there is an increase in the jejunal activity of leucine aminopeptidases and several dehydrogenases (Riecken et al., 1965).

Several dietary factors affect the digestibility of protein, such as the level of naturally occurring antinutrients (phytates, lectins, tannins, saponins, etc.), and enzyme inhibitors (Jenkins et al., 1985). Also, the digestibility of protein is affected by the origin of the food and its processing (Jones et al., 1962). Cooking may

denature protein allowing it to be more easily digested, while cooking can also bring about the formation of Maillard products, so decreasing the digestibility of some amino acids (Snook, 1973; Mason, 1984). The amount of a protein that is consumed may also affect some measurements of digestibility (Greeley et al., 1964; Gibson et al., 1976; Rerat, 1978). Low (1980a) has compared the digestibility values of amino acids from various protein sources, and diets, and has shown that the values for individual amino acids vary quite considerably with source.

Fibre type and level affect the faecal and ileal digestibility of protein (Murray et al., 1977; Just, 1980; Jorgensen et al., 1985; Low, 1985; Sauer and Ozimek, 1986), by decreasing digestibility (Just et al., 1985). There is an inverse relationship between cellulose content and the digestibility of organic matter including protein (Henry and Etienne, 1969). The inclusion of fibre in the diet may increase mucosal cell sloughing and mucus production, and fibre can adsorb amino acids and peptides preventing their absorption in the gut (Sauer and Ozimek, 1986). Some fibre components may influence the activity of pancreatic and intestinal enzymes, and therefore may influence the digestion of protein, as well as the secretion of gastrointestinal hormones (Kritchevsky, 1988). Also sugars and starch may affect the digestibility of protein, by affecting the absorption of amino acids (Zebrowska, 1980).

Other inhibitors of intestinal absorption also exist, and exert their effects by either altering the permeability of the mucosa, by affecting transport mechanisms, access to the mechanism, or the ability of the substrate to use the mechanism, or they may have an effect on the metabolic processes providing energy for normal function of the intestinal mucosa (Oxender and Whitmore, 1966; Sanford, 1967). Some inhibitors of absorption occur naturally in the diet, such as galactose which inhibits amino acid absorption possibly due to the toxic effects of galactose-1-phosphate (Newey and Smyth, 1964).

The nervous system has a mediating affect on gut function, and Golding _et al._ (1965) have demonstrated that absorption is impaired after vagotomy. Therefore, anything affecting the nervous system such as drugs, toxins, or emotional influences, may also affect the absorption of amino acids. Several other internal factors, such as blood flow, gut motility, stomach emptying and the gut microflora, influence absorption in the small intestine, so that any external factors influencing these may also alter the digestive and absorptive processes. When studying protein digestibility, it is important to be aware of the possible influencing factors and to attempt to control these wherever possible.

1.3. THE IN VIVO DETERMINATION OF AMINO ACID DIGESTIBILITY.

1.3.1. FAECAL VERSUS ILEAL DIGESTIBILITY.

Although the measurement of amino acid digestibility (i.e. release from protein and absorption) following the total collection of faeces has been the traditional approach with monogastric animals, it is becoming increasingly clear (Payne et al., 1968; Varnish and Carpenter, 1975; Moughan and Smith, 1985; Sauer and Ozimek, 1986) that the ileal method is preferable. In the latter case, amino acid digestibility is determined after collection of digesta from the terminal ileum by means of cannulation or fistulation of the terminal ileum, slaughter of the animal with collection of the ileal contents, caecectomy, or other methods (Wrong et al., 1981; Papadopoulos, 1985; Sauer and Ozimek, 1986).

It is considered that amino acid absorption is completed by the end of the small intestine (Nolan, 1985), and protein entering the large intestine is partly degraded by the microflora (see Section 1.2.3), with a net absorption of N mainly as NH_3. It does not appear that peptides or amino acids are absorbed as such in the large intestine in vivo (Low, 1980a; Wrong et al., 1981; Rerat, 1983; Nolan, 1985).

On average the absorption of amino acids measured in the faeces tends to overestimate absorption, due to a net disappearance of amino acids in the large intestine because of microbial activity (Just, 1980), although for some amino acids (methionine and lysine), a net synthesis may occur in the large intestine (Low, 1980a; Just, 1980; Sauer and Ozimek, 1986). The amino acids cysteine, arginine and tyrosine have also been reported to be higher in faeces than at the ileum (Just, 1980; Rerat, 1978). Particularly large differences in the amino acid content of ileal digesta and faeces may be seen for proline, threonine, glutamate, glycine and aspartate, which make up a large portion of the protein in mucoprotein and pancreatic juice. The digestibility in the large intestine varies according to the amino acid considered and the protein source. For example, the

difference in digestibilities between the ileum and faeces for glycine and proline in soyabean protein have been reported as 15 and 25 percentage units respectively and in ground wheat, 8 an 11% units respectively, and cracked wheat, 15 and 13% units respectively (Holmes et al., 1974; Sauer and Oximek, 1986). The greatest differences between ileal and faecal digestibility estimates for amino acids are seen for foodstuffs of low protein digestibility (Austic, 1983). Generally, the difference in digestibility measured by the ileal or faecal methods has been estimated to be 5-6% units, but differences of 10-20% units, have been reported, (Rerat, 1978).

It is concluded, therefore, that the digestibility of protein and amino acids is best determined after collection of terminal ileal digesta. The apparent ileal digestibility of each amino acid is calculated using the following equation:

$$1.1.\quad \text{Apparent A.A.}^{*1}\ \text{digestibility}\ (\%) = \frac{\text{A.A.I}^{*2} - \text{A.A.E}^{*3}}{\text{A.A.I}^{*2}} \times \frac{100}{1}$$

*1 A.A. = amino acid.
*2 A.A.I. = amino acid eaten (g/day).
*3 A.A.E. = amino acid excreted in ileal digesta (g/day).

The ileal method for determination of amino acid digestibility is open to error due to endogenous amino acids being present in the ileal digesta at relatively high levels. Therefore, apparent ileal digestibility coefficients may not be as useful as true coefficients (Low, 1980a). The true ileal digestibility of amino acids takes into account the endogenous contribution of amino acids in the gut and is calculated using the following equation:

$$1.2.\ \text{True A.A}^{*1}\ (\%)\ \text{digestibility} = \frac{\text{A.A.I}^{*2} - (\text{A.A.E}^{*3} - \text{E.A.A}^{*4})}{\text{A.A.I}^{*2}} \times \frac{100}{1}$$

[*1] A.A = amino acid

[*2] A.A.I. = amino acid eaten (g/day).

[*3] A.A.E. = amino acid excreted in ileal digesta (g/day).

[*4] E.A.A. = flow of ileal endogenous amino acid (g/day).

1.3.2. ENDOGENOUS PROTEIN EXCRETION FROM THE SMALL INTESTINE.

The nature of the diet may have an effect on the quality and quantity of endogenous N secreted in the gut (Sauer and Ozimek, 1986). The amount of N excreted when pigs were fed a protein-free diet was lower at the ileum than when pigs were fed a protein-containing diet (Buraczewska, 1980). The level of dietary protein, starch and lipid affect the specific activity of proteolytic enzymes (Corring, 1980). It has also been shown that total N in bile and the protein in pancreatic secretions are higher for less refined diets in pigs (Partridge et al., 1979; Sambrook, 1980). In humans (Roy et al., 1967; Rosenweig et al., 1971) the enzymes secreted by the small intestine are sensitive to dietary regulation. When using the ileostomate for the collection of ileal digesta there may also be different non-dietary contributions of amino acids due to ileal resection or microbial protein. Therefore, it may be especially important to correct for non-dietary protein.

Sources of endogenous N include secreted enzymes, mucoproteins, sloughed epithelial cells, plasma proteins and other nitrogenous compounds in the gastrointestinal secretions (Rerat, 1978; 1983; Nolan, 1985). Nasset (1965) has suggested that a considerable amount of N in the duodenal digesta is of endogenous origin. A study with humans (Nixon and Mawer, 1970b) suggests that after a protein meal, as much as 53% of the intraluminal protein in the small intestine may be of endogenous origin. Johansson (1975) has also shown that endogenous protein is about a third of the amount of that in the

diet. Other workers have reported that the endogenous N from proximal parts of the gut of experimental animals was equal to 10-50% of the N intake (Crompton and Nesheim, 1969; Zebrowska, 1971; 1973b; 1973c), while Adibi and Mercer (1973) reported little or no change in the free amino acid, peptide and protein fractions after a protein-free meal compared with the fasted state, in human ileal aspirates. The differences reported may arise due to different methods used to estimate endogenous loss.

The endogenous loss of N from the small intestine in adult humans has been estimated to be around 1g/d (Chacko and Cummings, 1988), while obligatory faecal N losses have been estimated to range from 0.25-1.1 g/d (Wrong et al., 1981), although values of around 0.5-0.7 g/d are frequently reported (Murlin et al., 1946; Schrimshaw et al., 1972; FAO/WHO, 1973). A large part of the faecal N is microbial, however the N in the small intestine is likely to be mainly from enzymes, mucus and mucosal cells. The ileal endogenous amino acid composition has been shown to vary according to the diet fed (De Lange, 1989), however the faecal endogenous amino acid composition remains relatively constant for a range of diets. Therefore, it may be more important to determine true digestibility at the ileum as opposed to the rectum.

There are several methods available for determining the levels of endogenous amino acids in the gut, although they tend to give varying results (Buraczewska, 1980; Zebrowska et al., 1983; Sauer and Ozimek, 1986). These include methods such as collection of ileal digesta after feeding the subject a protein-free meal, fasting, feeding the subject a meal with a small amount of a highly digestible protein such as egg, giving meals with different amounts of protein and extrapolating to zero protein intake, or collection of digesta after perfusion of the gut and before absorption occurs (Nixon and Mawer, 1970a; Adibi and Mercer, 1973; Zebrowska, 1973c, Kidder and Manners, 1978; Buraczewska, 1980; Low, 1980a; b; Sauer and Ozimek, 1986). A more recently developed method, the ^{15}N-isotope

dilution technique (Souffrant et al., 1986), involves the labelling of endogenous protein by constant infusion of the ^{15}N label. This then allows the endogenous contribution to be determined for the diet, for which the digestibility is being determined. This overcomes the problems of dietary conditions influencing the amount and composition of the endogenous secretions. This method, however, also has its inherent problems, as discussed by De Lange (1989), and at present can only be used to accurately determine total endogenous protein rather than individual endogenous amino acids flows.

There are also problems with all of the other methods. The perfusion of the gut with protein-free solutions to collect gut secretions before any absorption occurs, may not present a physiologically normal situation, as well as being technically difficult for application to human studies. The perfusate is 'isotonic' saline, however the saline may not be isotonic with the digesta in all regions of the gut and this will probably affect the endogenous secretions (Buraczewska, 1980). The method of feeding different levels of protein (the regression method) with extrapolation to zero protein assumes that the endogenous protein contribution is constant at all levels of protein intake, especially low and zero intakes, which it may not be (Carlson and Bayley, 1970; Sauer and Ozimek, 1986). The method involving feeding a highly digestible protein-source is dependent upon the validity of the assumption that all of the protein is in fact digested and absorbed, and the collection of material after a fasting period does not allow for dietary stimulation of gut secretions and mucosal cell contributions. The mucosal cell contribution and the enzyme secretion may be enhanced by feeding a protein diet rather than a protein-free one (Sauer and Ozimek, 1986). Therefore the protein-free diet method can also be criticised as it assumes that the amount of protein secreted in the gut is independent of the amount of protein eaten (Papadopoulos, 1985). De Lange (1989) has shown that both the diet composition and the protein status of the subject affect the levels of endogenous

protein and the amino acid composition, before the ileum, although true ileal amino acid digestibilities based on the different determinations of endogenous loss, varied only slightly despite significant variation in the endogenous amino acid compositions. Results of the protein-free method and the regression method often agree well (Carlson and Bayley, 1970; Taverner et al., 1981; Leibholz, 1982). Results from the ^{15}N-isotope dilution technique, however, are usually lower (M. De Lange unpublished) but may represent minimal losses. Skilton et al. (1988) have shown that there is no significant difference between endogenous amino acid determinations by either the protein-free method, or feeding diets consisting of synthetic amino acids devoid of a single amino acid, in each case. This suggests that the protein-deplete state which has a marked effect on the metabolism of protein in the body does not affect endogenous secretions into the gut. Further work from this group (Butts et al., 1989; Darragh et al., 1989; Moughan and Rutherfurd, in prep.), however, has shown that feeding diets devoid of protein or peptides results in lower endogenous protein contributions, implying a direct effect of dietary protein on the gut to increase endogenous protein levels. In these latter studies, the protein-free feeding was carried out over a period of several days, and the difference may not be as great over a short period of several hours, as is often used in work with humans. If the effect of protein, increasing the endogenous protein levels, is direct, the shorter period of several hours may not allow for complete adaptation of the gut to a protein-free state. Although the protein-free method may underestimate endogenous protein contributions to the gut, it is a commonly accepted measure and at least makes some correction for the endogenous protein, and so may give more accurate estimates of digestibility. In work with humans, the low protein or protein-free methods have often been used for determining endogenous flows (Nixon and Mawer, 1970a; Schrimshaw et al., 1972; Adibi and Mercer, 1973), and these methods are the simplest, least time consuming, and least expensive.

When working with human subjects it is often more convenient, and acceptable, to feed only a single meal, especially when using a protein-free diet. This may also have the advantage that the gut has not had time to fully adapt to the protein-free state. If a protein-free diet is given to subjects as a single meal, it becomes most appropriate to collect only a sample of ileal digesta. However, when samples of digesta are taken, the flows of nutrients need to be related back to the dietary intake by reference to a non-digestible marker compound.

1.3.3. THE USE OF NON-DIGESTIBLE MARKERS IN DIGESTION STUDIES.

Non-digestible, non-absorbable markers are often included in diets where a sample of digesta is collected rather than a total collection made. The concentration of the marker in the digesta is used to relate back to its concentration in the diet, and therefore the amount or flow of amino acids at the ileum or faeces can be expressed in terms of the amount of food consumed. The flow of amino acids can be calculated using the following equation:

1.3. $$\text{Flow of amino acid } (g/gDM_I{}^{*1}) = A.A._E{}^{*2} \times \frac{marker_I{}^{*3}}{marker_E{}^{*4}}$$

*1 DM_I = dry-matter eaten.
*2 $A.A._E$ = amino acid excreted in digesta (g/g dry-matter).
*3 $marker_I$ = concentration of marker in diet (g/g dry-matter).
*4 $marker_E$ = concentation of marker in digesta (g/g dry-matter).

For a marker to be effective, it should be completely recovered at the site of sample collection. A marker substance must also be carried through the digestive tract at the same rate as the flow of the substance being investigated. Differential transit rates may be a problem. For example, soluble markers may move with the liquid phase of the digesta ahead of the solid phase, and insoluble markers

may separate out from the digesta during stomach emptying or movement through the gut. Several different markers have been used in human and animal studies, including chromic oxide, polyethyleneglycol, glass beads, plastic tubing, radioactive markers, dyes such as carmine, and lignin (Kotb and Luckey, 1972; Branch and Cummings, 1978; Hesp et al., 1979; Bacon, 1980).

A further problem with using marker substances, especially in human studies, is the acceptability of the compound. Some markers colour the food and digesta, and may not be accepted by the subjects. Radioactive markers such as ^{51}Cr and ^{131}Ba which are often used in animal studies, also may not be acceptable in human studies. The best markers to use for overcoming at least some of these problems are internal markers, or substances which occur naturally in the food but are not digestible. Lignin and acid-insoluble ash occur naturally in the diet and have been used in animal studies (McCarthy et al., 1974), but because of the refined nature of the human diet, the levels of these substances in human diets are too low to measure. There have been reports of variable recoveries of lignin from human and animal work, which may be partly due to the chemical determination of lignin (Kobt and Luckey, 1972). A suitable solution may be to use acid-insoluble ash, as Celite, added to the diet as a marker. Celite (which is fully recovered analytically as acid-insoluble ash), is a fine white powder, consisting of natural silicates. To date, however, it has not been used as a marker in human studies, and work needs to be conducted to determine its usefulness in studies on protein digestibility involving human subjects.

To determine amino acid digestibility coeffients in humans it is necessary to be able to collect the appropriate samples. The collection of faeces can be made by using normal healthy individuals, and the collection of ileal digesta can most easily be achieved by using subjects who have undergone an ileostomy operation due to disease of the large intestine, such as Crohns disease or ulcerative colitis.

1.3.4. THE ILEOSTOMY MODEL.

The ileostomy operation involves exteriorisation of the end of the small intestine after it has been divided, through a small incision in the abdominal wall. The end of the intestine is turned onto itself and the cut end is sutured to the skin, forming a stump of intestine called the stoma, which is usually 2.5-4.0 cm long (Fig.1.2). An appliance is fitted over this stoma, and a bag is attached for collection of the ileal output. The remaining large intestine is usually resected, and there is also often some resection of the small intestine (Brooke, 1952; 1969).

Large scale resection of the rat small intestine has been shown to influence the gut by eliciting an hormonal response which increases the rate of turnover of cells and the length of the villi (Loran and Althausen, 1960). This may affect not only the endogenous protein contribution to the gut, but also its absorptive capacity. To what extent this occurs in human ileostomates is not known. Resection of the small intestine can also lead to compensatory changes in the residual gut (Dowling, 1967). Booth et al. (1959) have reported that there is a slight positive enlargement of the jejunum after ileal resection in rats. Dowling and Booth (1966) have also shown that human patients with large scale small intestinal resection (at least 1.5 m) absorbed 51-67 mg glucose/25cm jejunum/min compared with controls who only absorbed 38-56 mg.

When considering the ileostomate as a model for the normal intact human, another potential problem is that ileostomates suffer from chronic Na^+ and water depletion (Clarke et al., 1967a; Haalboom et al., 1983), which can alter the volume and composition of the ileal effluent. A decreased intake of salt will decrease the volume of ileal effluent and Na^+ concentration, and increase the K^+ concentration (Clarke et al., 1967b). How this affects the digestion of protein and other food components is not fully known. Kramer

(1966) found that increasing levels of salt intake had little effect on the N content of the ileal effluent. Therefore this effect should have little influence on the determination of protein and amino acid digestibility when using the ileostomate.

Another potential problem when using the ileostomate for ileal digesta collection is the effect of the microflora in the gut. After an ileostomy, the distal ileum becomes colonised with a characteristic microflora which is stable after one to three weeks (Vince et al., 1973). The microflora may influence the turnover of small intestinal mucosal cells. Studies in germ-free animals have shown that the turnover of cells is about half that in normal animals (Creamer, 1967). If the levels of microorganisms are higher than normal this too may affect the turnover of intestinal cells, which may have an effect on the levels of endogenous protein in the gut, and therefore estimates of apparent protein digestibility.

The total number of organisms recovered from ileostomy output is about 80 times that in normal ileal contents (Gorbach et al., 1967). Vince et al. (1973) found that the total number of aerobes were the same, but yeasts and gram positive cocci were greater in the ileostomy effluent than normal terminal ileal contents.The effect of the microflora on the determination of protein digestibility will depend on the numbers of microorganisms and types present in the small intestine, as well as the rate of passage of digesta through the small intestine, which determines how long the microflora have to act on the protein in the digesta. The transit of digesta through the small intestine is rapid, and has been estimated by Dillard et al. (1965) as being 3.6-5.9 cm/minute. The rate is similar for ileostomates and normal subjects (Chapman et al., 1985), and the effect of the microbial activity on the determination of protein digestibility is likely, therefore, to be small. Any effect of microbial activity on protein digestiblity will tend to make the ileal determinations closer to the faecal determinations, and so any differences found between the two methods when using the

ileostomate, should be regarded as minimum differences.

Studies investigating the numbers and types of microorganisms in human ileostomy output to date, have involved total counts of the microorganisms (Gorbach et al., 1967; Vince et al., 1973). Other methods, however, are available for indicating the presence of microorganisms in digesta samples. These methods involve measuring the levels of certain substances, which act as bacterial markers.

a. Bacterial Markers.

Bacterial markers include such naturally occurring substances as: the nucleic acids, DNA and RNA: the amino acid, diaminopimelic acid (DAP): 2-aminoethylphosphonic acid (a protozoal marker), or methods such as the microbial incorporation of radioactive labels ^{15}N and ^{35}S (Ling and Buttery, 1978; Cummings, 1984). Several studies in ruminant work comparing these markers have shown that estimates of microbial protein obtained can vary widely (Ling and Buttery, 1978; Siddons et al., 1982; McAllan and Smith, 1984; Whitelaw et al., 1984), with the differences between methods not being consistent between studies (Ushida et al., 1985). Therefore, when attempting to estimate bacterial levels in digesta samples indirectly, it would seem appropriate to include several different methods. Some of the above methods have been used in studies with monogastric animals (Salter and Coates, 1971; Mason and Palmer, 1973; Wolstrup et al., 1979; Fleming and Wasilewski, 1984), but have not been used in studies involving human subjects to any significant extent (Cummings, 1984).

Diaminopimelic acid occurs in the bacterial cell wall mucopeptide, and is not found in plant or animal cells. The only disadvantage of using DAP to predict the concentration of bacteria in digesta is that different species of bacteria vary in their DAP content (Synge, 1953; Dufva et al., 1982), and

dietary conditions may influence the relative levels of bacterial species present, and therefore the DAP content in the digesta (Czerkawski, 1974; Ling and Buttery, 1978). This may not be a problem when comparing levels of DAP in digesta samples, collected after the same diet has been fed to the animals or subjects involved (Czerkawski, 1974). A comparison, however, between ileal digesta and faecal samples, or between two different species does not take into account the different bacterial species present in different parts of the gut, or in the digestive tracts of the two species, and may, then, provide misleading results.

The nucleic acids, DNA and RNA, are often used in ruminant work as bacterial markers. They are either measured separately or as total nucleic acids. Nucleic acids are present in animal and plant cells, and will therefore be present in the diet, and the mucosal cells in the gut. Therefore the use of nucleic acids to indicate the levels of bacterial cells present in digesta samples relies on the assumption that dietary nucleic acids are almost completely digested and absorbed in the gut (Nolan, 1985), and that background levels of nucleic acids from sloughed-off mucosal cells in the gut are constant throughout the gut for any one diet. It has been shown that the microbial RNA content is not constant per unit of cell mass, and varies with specific growth rates of microorganisms, and with the diet (Bates et al., 1985) Bates et al. (1985) reported that for ruminant bacteria, the DNA:protein is largely independant of growth rate in vitro, and they suggest DNA:protein would be a better indicator of bacterial content than RNA:protein. Once again, the variation in nucleic acid levels may not be as great when comparing between samples collected after feeding the same diet to the subjects.

2-Aminoethylphosphonic acid is commonly used as a protozoal marker, although it has been found to be present in diets and

bacteria (Ling and Buttery, 1978; Whitelaw et al., 1984), as well as a range of other materials including human tissue (Alhadeff and Daves, 1971). This has led to overestimation of microbial protein and considerable variation in measurements.

Another method that could be used to indicate the level of microbial activity in digesta samples, is the measurement of the extent of fibre digestion. Dietary fibre can be defined as carbohydrate and some non-carbohydrate components that are resistant to mammalian digestive enzymes (Trowell et al., 1976). It can therefore be assumed that any digestion of fibre that occurs, is due to microbial fermentation.

There are four main components which make up dietary fibre. Cellulose, which is very insoluble, is the main structural component of plant cell walls. Hemicellulose is a heterogenous group of sugar chains, which exhibits a wide range of solubilities. Pectin is composed mainly of D-galacturonic acid, it is highly soluble, and is found in cell walls and as intercellular 'cement'. Lignin is a highly complex, three dimensional non-polysaccharide polymer. It is generally inert, insoluble and resistant to bacterial degradation (Schneeman, 1986). Analytically, the lignin fraction includes Maillard products where these are present (Van Soest, 1984). The degree of degradation of individual fibre components in the intestinal tract varies. Pectins, mucilages and gums appear to be completely broken-down by the end of the large intestine, but cellulose is only partly broken-down (Schneeman, 1986).

It has long been accepted that digestion of dietary fibre only occurs in the large intestine, and that there is no evidence for significant breakdown occurring in the stomach or small intestine of humans (Sandberg et al., 1981; Cummings, 1985; Englyst and Cummings, 1985; 1986; Sandstrom et al., 1986). These studies and others investigating fibre digestion, used

the ileostomate as an *in vivo* model (Holloway *et al.*, 1978; 1980; 1983; Sandberg *et al.*, 1981; Englyst and Cummings, 1985; 1986; Sandstrom *et al.*, 1986). In many of these studies the effect of microflora on fibre digestion before the ileum was considered to be slight (Holloway *et al.*, 1980; Sandberg *et al.* 1981; Sandstrom *et al.*, 1986), although some workers (Holloway *et al.*, 1980; 1983) reported less than 100% recovery of various fibre components in the ileostomy output, implying that bacterial degradation had occurred.

The discrepancies in fibre recovery may have been due to microbial activity occurring in the ileostomy bag. In most of the above studies it was specified that the bags were emptied regularly, for example every 2-3 hours, and the contents immediately chilled or frozen, to minimise the effects of microbial activity. However, some studies did not state what procedure was used in the collection of samples, or if the suggested procedure was adhered to rigidly. The studies by Sandberg *et al.* (1981) and Sandstrom *et al.* (1986) both stated that ileostomy bag contents were emptied every two hours and immediately frozen. Sandstrom *et al.* (1986) reported almost complete recovery of non-starch polysaccharides (93-99%) and phytic acid (91-100%) in ileostomy output for meat and soy-based diets. Sandberg *et al.* (1981) claimed that no significant breakdown of fibre had occurred before the ileum, but only recovered 85% of the hemicellulose in bran in the ileostomy output. Holloway *et al.* (1978; 1980; 1983), did not state how often the digesta were collected and frozen. However, they reported no degradation of hemicellulose or cellulose in bran occurring before the ileum in one study, but considerably less than 100% recovery of hemicellulose and pectin in the latter two studies.

Additional information on the digestion of fibre components can be gained from studies using experimental animals such as the

pig and the rat (Van Soest et al., 1983). Several workers have suggested that the pig (Fleming and Wasilewski, 1984; Low, 1985) and the rat (Nyman et al., 1986) may be suitable models for humans for fibre digestion. In a study using the pig (Sambrook, 1979), the digestion of acid-detergent fibre at the terminal ileum was determined for different diets, and found to range from 8-50%. Millard and Chesson (1984) also used the pig, and reported up to 50% loss of various fibre components, before the ileum. Kass et al. (1980) reported 38% digestion of hemicellulose before the ileum of the pig, with complete recovery of cellulose. Keys and DeBarthe (1974) also used the pig, and reported 5-10% loss of hemicellulose before the ileum, for most diets, but up to 50% for one diet. For most diets cellulose was completely recovered, although for one diet there was up to 33% loss of cellulose, before the ileum. It appears, therefore, that a limited degree of microbial breakdown of fibre may occur before the end of the ileum in the ileostomate and the intact pig. The determination of fibre digestibility may, therefore, be a useful indicator of the extent of bacterial activity, in the small intestine.

The bacterial population of the normal human small intestine is likely to be lower than in the ileostomate, and so when using the ileostomate in digestibility studies, as a model for the intact human, it would be useful to have some information on the extent of colonisation and the activity of the microflora. The above discussed markers are useful for this purpose.

1.3.5. STUDIES OF PROTEIN DIGESTIBILITY USING HUMAN SUBJECTS.

In humans, amino acid, N and protein excretion and digestibility have traditionally been measured after collection of faeces (Schrimshaw et al., 1972; 1983; Cummings et al., 1979; Forsum et al., 1981; Wayler et al., 1983; Istfan et al., 1983; Young et al., 1984; Morales and Graham, 1987), and to date there have been only a

few attempts (Gibson et al., 1976; Holgate and Read, 1983; Sandstrom et al., 1986) at measuring protein digestibilities by the ileal method. Information on the size of the human large intestine relative to the rest of the gut, and the transit rate of material through the tract, would support that ileal/faecal differences for amino acid digestibility may also occur in humans.

Sandstrom et al. (1986) used ileostomates to investigate the small intestinal absorption of N from soy and meat protein-based diets, and reported apparent N digestibilities of 75-80%, and true N digestibilities of 80-85% (assuming an obligatory faecal loss of 8.8mg N/kg body weight). Earlier studies (Schrimshaw et al., 1983; Wayler et al., 1983; Istfan et al., 1983; Young et al., 1984) with normal subjects, and assuming the same loss of N, reported true faecal digestibilities of 90-98% for soy protein-based diets. Other studies with ileostomates have reported apparent digestibilities of 87-91% for animal protein-based diets (Sandberg et al., 1981; 1982), and Holgate and Read (1983) found that 83% of the protein in a single meal was digested and absorbed before the ileum.

Sammons (1961) also conducted a study with human subjects, and found that the amount of N excreted/day in ileostomy output and faeces for subjects on the same diet was 2.7g and 1.8g respectively. Gibson et al. (1976) fed 2 levels of protein intake to ileostomates and normal subjects, and reported ileal and faecal protein digestibilities for the lower level of 95.5% and 97.0% respectively, and for the higher level of 97.4% and 98.4% respectively. These studies suggest that there is a difference between N digestibilities determined by the ileal and faecal methods in humans, and so it follows that there will probably also be a difference for the determination of amino acid absorption between the two methods. From the limited information available, it seems as though faecal digestibility coefficients overestimate the extent of protein digestion in humans as has also been shown to be the case for domestic animals.

1.3.6. THE DETERMINATION OF AMINO ACID VERSUS NITROGEN DIGESTIBILITY.

In most human digestibility studies, workers have only been interested in N or protein digestibility, and have not determined amino acid digestibility. In protein nutrition, knowledge about individual amino acids is important, because humans have different requirements for individual amino acids, and determination of the extent of digestion and absorption of individual amino acids in foodstuffs may differ from the digestibility of crude protein.

The pattern of amino acid absorption in the small intestine generally follows that of N absorption. However, Zebrowska (1973c) reported that the relative amounts of the free amino acids in ileal digesta of pigs differed from the amino acids in the dietary protein by different degrees, depending on the diet fed. It was suggested that this was probably due to the different rates of release and absorption of amino acids during digestion. The digestibility of individual amino acids, therefore will be different from each other and the overall N digestibility (Nielsen, 1971; Moughan and Smith, 1985; Sauer and Ozimek, 1986). Proline and glycine, for example, are often present in the digesta in higher proportions than are total N and other amino acids, due to a high proportcn of these amino acids in endogenous secretions (Adibi and Mercer, 1973; Rerat, 1978). Zebrowska (1973c) has shown that threonine, serine and alanine are absorbed more slowly than other amino acids, and so may have lower digestibility values than total N. Individual amino acid digestibility values may be required when formulating special diets, or when determining the quality of protein sources or mixed diets.

A considerable amount of work needs to be done in assessing these values for human foods and diets, and it is becoming increasingly difficult to carry out experimentation using human volunteers. To allow for routine analysis of human diets, therefore it is desirable that a model animal be used.

1.4. THE ROLE OF MODEL ANIMALS.

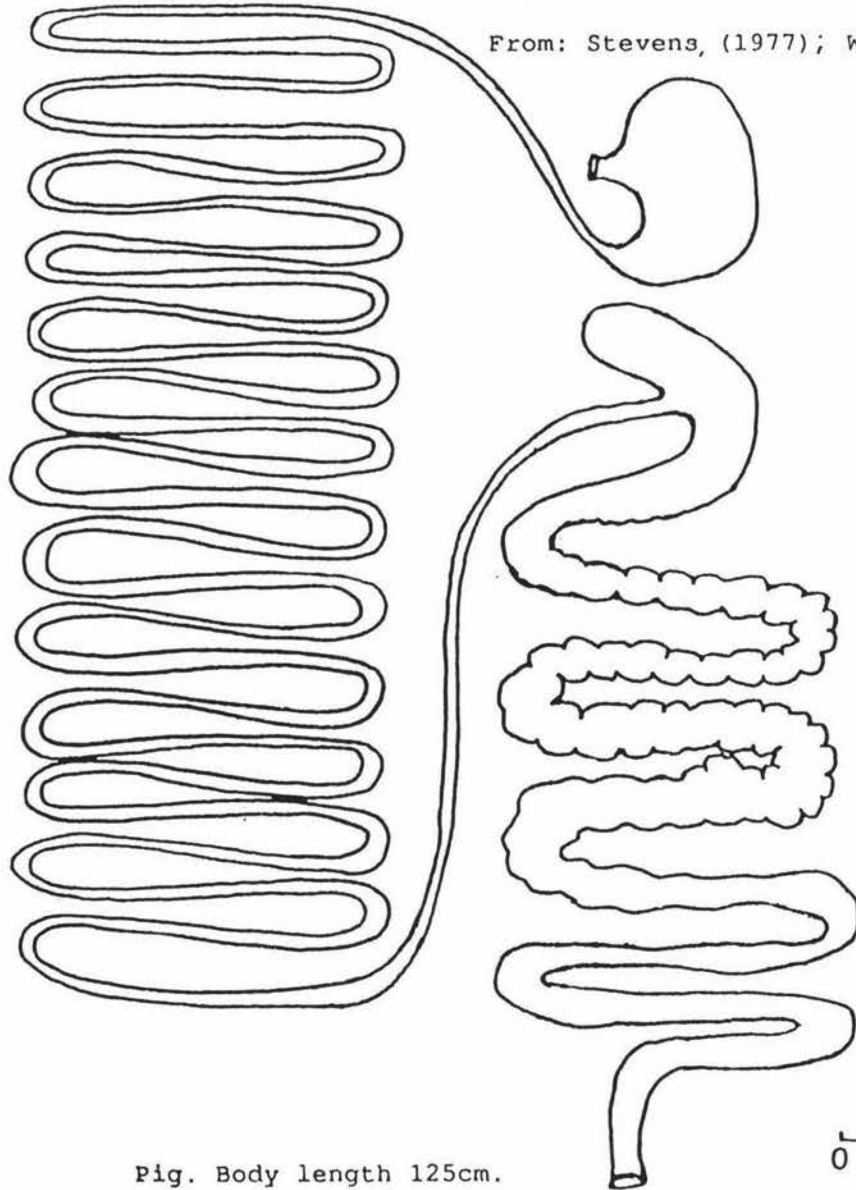
Experimental animals are often used as models in human biomedical research, not only because it is becoming more difficult to use human subjects for ethical reasons, but also because far more control over experimental conditions can be achieved by using animals. Several workers have claimed that the pig is an acceptable model for human nutrition studies (Pond and Houpt, 1978; Dodds, 1982; Rerat, 1983; Ratcliffe, 1985; Miller and Ullrey, 1987), and the pig is frequently used as a model for research in human nutrition and related clinical disorders (Pond and Houpt, 1978; Miller and Ullrey, 1987; Tumbleson, 1986). Much is known about the pig, and in addition to this the pig is a rapidly growing animal and a litter-bearing species, so it matures rapidly and allows for experiments to be carried out on genetically similar animals. The pig is also easily obtained and relatively inexpensive, as well as being easy to confine, making it a better choice than primates (Laplace, 1981).

In addition to logistical reasons for choosing the pig as a model, the pig is similar to humans in several aspects related to nutrition. The pig is omnivorous and is an intermittent feeder, (ie. eats its food as meals). The nutrient requirements of the two species are also very similar, with the 25kg pig having similar energy requirements to the adult human. Also, the pig exhibits similar responses to many nutritional disorders (Pond and Houpt, 1978). Neither pigs nor humans practice coprophagy, as is the case for the rat. Further, the two species have a similar gastrointestinal anatomy (Fig.1.4), physiology and metabolism.

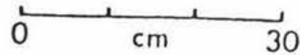
The following sections, attempt to examine in detail aspects of digestive anatomy, physiology, biochemistry and microbiology in pigs and humans to allow clearer definition of the suitability of the pig as a model.

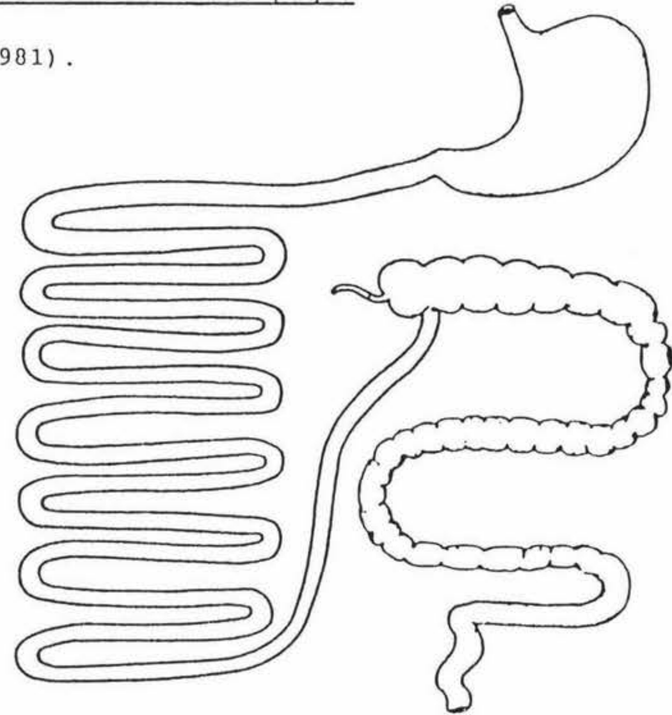
Figure 1.4. The digestive systems of adult humans and pigs.

From: Stevens, (1977); Wrong *et al.*, (1981).



Pig. Body length 125cm.





Human. Body length 180cm.

1.4.1. THE STOMACH.

The stomach has the same basic functions in the two species, acting as a reservoir to receive and store the ingested food, while mixing it and mechanically breaking it up, and preparing it for digestion later in the tract. Some digestion does occur in the stomach due to the action of salivary amylase, and gastric pepsins. The stomach capacity of each species is given in Table 1.1.

In both species the stomach consists of 3 main regions, the cardiac gland region (fundic), proper gland region (body), and the pyloric gland region. In the pig the fundus region has a flattened conical diverticulum distinguishing it from the stomach of other monogastric species (Nickel et al., 1973). The pig's stomach is also different in that it has a small non-glandular part (esophageal region), around the area that the esophagus enters the stomach.

The stomach wall consists of the same four layers in both species. The mucosal layer is made up of epithelial connective tissue, lamina propria and muscularis mucosae. The submucosa layer consists of connective tissue containing blood vessels, lymphatics and a nerve plexus. The muscle layer consists of 3 separate layers of visceral muscle and a nerve plexus. The serosal connective tissue covered by mesothelium makes up the fourth layer.

There are 3 types of glands present in the stomach mucosa. The gastric glands secrete water, electrolytes, mucus, pepsinogen, hydrochloric acid (HCl) and intrinsic factor. In humans, the gastric glands cover 70-80% of the mucosa, while in the pig they only occupy about 33% of the mucosal surface. The pyloric glands secrete mucus and gastrin, and in humans they cover about 15% of the surface, and about 33% in the pig. The cardiac glands secrete mucus, and cover 5-15% of the surface in humans, and about 33% in the pig (Nickel et al. , 1973; McMinn and Hobdell, 1974).

Table 1.1. The relative sizes and proportions of different parts of the digestive tract of the adult human (65kg) and the growing pig (25kg).

Organ		Human*2	Pig*1
Stomach	Capacity (l/kg BW*3)	0.014-0.021	0.088
	Weight (g/kg BW)	-	13.8
	Relative capacity(%)*4	16.0	29.0
Small intestine			
	Duodenum length (cm/kg BW)	0.4	3.0
	Jejunum length (cm/kg BW)	4.0	26.0
	Ileum length (cm/kg BW)	5.0	39.0
	Total capacity (l/kg BW)	0.10	0.32
	Relative capacity (%)*4	67.0	33.0
Large intestine			
	Length (cm/kg BW)	2.00	15.0
	Relative capacity (%)*4	17.0	38.0
Weight large bowel contents	(% weight of animal)	0.43	4.80
Intestinal length:body length		5:1	14:1

*1 Dillard *et al.*, 1965; McMinn and Hobdell, 1974; Bockus, 1976a; b; c; Thompson, 1977; Wrong *et al.*, 1981.

*2 Nickel *et al.*, 1973; Kidder and Manners, 1978; Wrong *et al.*, 1981.

*3 BW = body weight.

*4 % of total gut.

The composition of the gastric secretion is a function of the rate of secretion, which is lowest after a fasting period. Following a fasting period, there is a small volume of gastric juice secreted in the pig, with a low pH but high proteolytic activity and high content of mucus (Noakes et al., 1968). In humans, following a fasting period, there is also a small volume of gastric secretion, which has a pH of around 0.9-1.2, low electrolyte concentration, and a low pepsin content (Bockus, 1976a). The secretion of gastric juice is stimulated by the ingestion of food, through three phases. The cephalic phase occurs while food is in the mouth, through taste and chewing, and smell can also act as a stimulant, ending when food is swallowed. The gastric phase begins when food enters the stomach, and continues until the stomach is empty, with the main stimuli being distention of the stomach, the presence of food against the mucosa, and products of gastric digestion. The intestinal phase begins when food enters the duodenum, and is stimulated by distention and the presence of digestion products (Davenport, 1982; Magee and Dalley, 1986). It has been estimated that the total daily gastric secretion has a pH of around 2 in both species, with the pig producing approximately 74-150 ml/kg dry-matter eaten/kg body weight, and humans producing approximately 55-115 ml/kg dry-matter eaten/kg body weight (McMinn and Hobdell, 1974; Bockus, 1976a; Pond and Houpt, 1978; Davenport, 1982; Zebrowska et al., 1983). The volume secreted will depend on the diet eaten, and other stimuli (Bockus, 1976a).

The rate of gastric emptying of digesta will influence the rate of digestion in the whole tract, and is determined by the nature of the food ingested, the acidity of the contents, and the volume of material in the stomach (Bockus, 1976a; Rerat, 1981; Magee and Dalley, 1986). A liquid meal passes through the stomach faster than a solid meal, and food particles must be reduced to a particular size (of about 2mm), before passing through the pyloric sphincter into the duodenum, although larger particles will eventually pass through (Magee and Dalley, 1986). The chemical composition of the

ingested food also influences the rate of emptying. For example, a high carbohydrate meal leaves the stomach faster than a high protein meal, which leaves faster than a high fat meal (Bockus, 1976a). Also the pH of the stomach contents affects the rate of emptying in humans. The lower the pH, the slower is the emptying, partly due to an inhibitory mechanism produced by acid in the duodenum, and is probably a protective mechanism (Bockus, 1976b; Magee and Dalley, 1986). This mechanism, however does not appear to occur in the pig (Rerat, 1981).

Gastric emptying begins as soon as the food enters the stomach, with a liquid meal being completely emptied within 1-2 hours in adult humans (Hunt and Spurrell, 1951), a semi-solid meal taking around 4 hours (Malagelada et al., 1976), and a solid meal taking up to 9 hours to leave the stomach (Davenport, 1982). In the pig, gastric emptying begins as soon as the food enters the stomach (Laplace, 1981), with 72% of 2mm particulate markers being emptied within 12h (Clemens et al., 1975). With a semi-purified diet, up to 30% of the dry-matter ingested may leave the stomach in the first 30 minutes, but after that complete emptying may take up to 24 hours (Cuber and Laplace, 1979a; b), and large particles may take even longer (up to 48h) to empty (Clemens et al., 1975). The apparent differences between the two species may be due to the different diets fed in these studies, which would affect the rate of emptying. The pig probably received a less refined diet than humans, and the pig may not chew its food as extensively as humans do. Further, the study by Clemens et al. (1975) used particulate markers, so the larger particles (1 and 2cm) would take a long time to leave the stomach, however if they were food particles they may be digested and mechanically broken up during this time and so would probably leave sooner. The rate of gastric emptying may influence digestion in the small intestine, and the rate of passage through the rest of the gut.

1.4.2. THE SMALL INTESTINE.

The small intestine in both species is made up of three parts, the duodenum, jejunum and ileum. The lengths of these parts, and the total and relative capacities of the small intestine are given in Table 1.1.

The duodenum is made up of four parts, the superior (first) part, descending (second) part, transverse (third) part, and the ascending (fourth) part. In humans, the bile and pancreatic ducts enter the second part of the duodenum about 8-10cm from the pylorus and the accessory pancreatic duct enters about 2cm before this site. In the pig, the bile duct enters about 2cm from the pylorus and the pancreatic duct enters about 14cm from the pylorus.

The intestinal wall is made up of the same 4 basic layers as in the stomach. The epithelium forms glands called the crypts of Lieberkuhn, by forming finger-like projections called villi. In adult humans there are 22-40 villi/mm^2 in the duodenum and jejunum, and 18-31 villi/mm^2 in the ileum (Bargmann, 1962, cited by Pearse and Riecken, 1967). The villi are longest in the duodenum in humans, being about 1mm long, and only half that near the ileocaecal valve. In the mature pig the height of a villus in the small intestine is approximately 0.5mm (Dellmann and Brown, 1976), but is longer in the jejunum than the duodenum or ileum (Sloss, 1954).

The epithelial cells or columnar cells have microvilli which are about 1u long and 0.1u wide, with about 1700/cell in adult humans (Brown, 1962; Trier and Rubin, 1965), and 0.6-1.3u long and 0.1u wide in the young pig jejunum (Kenworthy et al., 1967). The microvilli form the striated border of the upper part of the absorptive epithelium, or the brush border. A study in humans (Lipkin et al., 1963) has shown that the mean regeneration time for epithelial cells in the ileum is 24-36 hours, as in the stomach and large intestine. In the young pig the turnover of the epithelium is approximately every 24-48 hours in the small intestine (Moon, 1971).

The columnar cells produce enterokinase and alkaline phosphatase, which are secreted into the lumen of the gut, and other enzymes which act within the cells or adhere to the outside of the cells at the brush border, such as disaccharidases, a lipase and peptidases (Smith _et al._, 1983). The epithelium is also made up of goblet cells which secrete mucus, and endocrine cells which produce hormones. There are also paneth cells which are found at the base of the crypts, in humans, and contain enzymes, although their exact function is not known (McMinn and Hobdell, 1974). There are no paneth cells in the pig small intestine (Pearse and Riecken, 1967), although the significance of this in relation to protein digestion and absorption is unknown, due to the unknown function of the cells.

The submucosa layer has Brunner's glands within it, and these glands secrete mucus. The muscle in the small intestine consists of only 2 separate muscle layers. The small intestine is also characterised by prominent folds or _valvulae conniventes_ in the duodenum and jejunum, with fewer in the ileum.

The pancreas secretes 0.02-0.04 ml/kg body weight/kg dry-matter eaten in the adult human (McMinn and Hobdell, 1974; Bockus, 1976b; Magee and Dalley, 1986) and approximately 0.05 ml/kg body weight/kg dry-matter eaten in the growing pig (Partridge _et al._, 1982). The volume secreted varies however, with the diet eaten (Bockus, 1976b; Zebrowska _et al._, 1983). The composition and secretion rate of the pancreatic juice of humans and the pig are given in Table 1.2. The composition of the pancreatic secretion is similar in both species, and the secretion rates of the various components are also similar, except there appears to be a greater amount of protein secreted each day for humans than pigs. However, the pig has a higher dry-matter intake than humans and this may compensate for this difference. The amounts of each enzyme secreted are also approximate values as different studies use slightly different methods for measuring the activities of each enzyme.

Table 1.2. The secretion rate of some of the components of pancreatic juice in the adult human (65kg) and the growing pig (25kg).

Component (mg/kg/kgDM) [*1]	Human [*2]	Pig [*3]
Protein	109.6-284.4	239.7-415.0
Nitrogen	23.4-50.2	46.5-82.3
Ash	200	300
Sodium	120	110-285
Potassium	8	8-35
Calcium	6	1-5
Phosphorus	2	0.05
Trypsin [*4]	0.9-1.7	2.2-2.6
Chymotrypsin [*4]	2.7-4.8	1.6
Lipase [*4]	60	12
Amylase [*4]	7.4-14.8	19.4

[*1] mg/kg body weight/kg dry-matter eaten.

[*2] Geigy Scientific Tables, 1970; Goldberg and Wormsley, 1970; Smith et al., 1983.

[*3] Low, 1982b; Partridge et al., 1982; Zebrowska et al., 1983.

[*4] U x 10^{-3}/kg/kgDM, U are activity units which were determined using similar substrates in both species for each enzyme.

The major enzymes found in the small intestine from pancreatic juice and from intestinal secretions are summarised and compared between the two species in Table 1.3. The pH optima for enzymes active in the small intestine are between pH 6.5-9.0, and the pH of various sites in the digestive tract are given in Table 1.4. The same enzymes are found in both species, although pigs are often lactase deficient, as are some human populations. The range of pH values are also similar throughout the gut for both species.

The digestion of dietary protein and absorption of amino acids has been discussed in Section 1.2. The specificity of proteases is essentially the same in humans and the pig. The nature and specificity of most of the intestinal disaccharidases are similar in humans and the pig (Dahlqvist, 1981). The site of activity of some saccharidases differs between the species. For example, in the pig maltase, isomaltase, and sucrase predominate in the lower small intestine, and lactase, trehalase and cellobiase predominate in the upper part, while in humans lactase activity is greater in the lower small intestine, and sucrase activity is greater in the upper parts (Dahlqvist, 1961; Newcomer and McGill, 1966).

Dietary fat is digested in the small intestine by lipases secreted from the pancreas and intestine, while a salivary lipase may also contribute to digestion. In the pancreatic juice there are at least three lipolytic enzymes (Davenport, 1982). The first, lipase, has a wide specificity but requires the dietary lipid to be emulsified, which is aided by bile and colipase. The second enzyme hydrolyses esters of alcohols and requires bile salts for its action, while the third enzyme hydrolyses water soluble esters. Pancreatic juice also contains phospholipase A which is activated by trypsin (Davenport, 1982). Fat digestion appears to be similar in man and the pig (Kidder and Manners, 1978).

Table 1.3. The digestive enzymes present in the adult human (65kg) and the growing pig (25kg).

Enzyme	Human*1	Pig*2
Trypsin	+*3	+
Chymotrypsin	+	+
Carboxypeptidases A and B	+	+
Elastase	+	+
Lipase	+	+
Amylase	+	+
Maltase	+	+
Isomaltase	+	+
Sucrase	+	+
Trehalase	+	+
Lactase	±*3	+
Cellobiase	+	+
Phospholipase	+	+

*1 Davenport, 1982; Magee and Dalley, 1986.

*2 Pond and Houpt, 1978; Corring, 1980; Dahlqvist, 1981.

*3 + = present, ± = sometimes present.

Table 1.4. The pH of various regions of the digestive tract in the adult human (65kg) and the growing pig (25kg).

Site		Human[*1]	Pig[*2]
Stomach		2	1.5-3.0
Small intestine	1[*3]	5.8-7.0	5.5-6.6
	2	6.0-8.0	5.9-7.8
	3	7.0-8.0	6.6-8.4
Large intestine		5.6-8.0	5.6-7.2
Faeces		6.1-7.0	6.4-7.8

[*1] Borgstrom et al., 1957; Nixon and Mawer, 1970b; Meldrum et al., 1972; Wrong et al., 1981; Wrong and Vince, 1984.

[*2] Williams Smith and Jones, 1963; Clemens et al., 1975.

[*3] The small intestine approximately at the beginning, in the middle and at the end respectively.

The pig secretes approximately 0.03-0.07 l of bile/kg body weight/kg dry-matter eaten (Corring, 1980; Low, 1980a), and the adult human secretes approximately 0.03 l of bile/kg body weight/kg dry-matter eaten (Davenport, 1982). The composition of bile is compared between the species in Table 1.5, and for most components the secretion rates are very similar for the two species.

Adrian and Bloom (1981) have shown that the release of gastrointestinal hormones in the pig and man is qualitatively the same, with small differences in quantity. The gastrointestinal regulatory peptides have been isolated and purified for the pig, as well as for humans, and therefore species similarities have been demonstrated. It has been suggested (Adrian and Bloom, 1981) that the pig would serve as a suitable model for humans for the study of the physiology of these peptides.

The rate of passage of digesta through the small intestine is rapid, and Dillard et al. (1965) reported that the rate of movement in the middle of the human small intestine was 3.6-5.9 cm/minute, which means that the time taken to move through the entire intestine is approximately 100-170 mins. The time taken for a barium meal to reach the end of the ileum, after leaving the stomach in humans has been reported to be 80-190 minutes (Magee and Dalley, 1986), and within 4-5 hours all of an average sized meal has reached the terminal ileum (McMinn and Hobdell, 1974; Magee and Dalley, 1986). The rate of passage of digesta in the small intestine of the pig is also rapid, and Clemens et al. (1975) found that there was no accumulation of fluid, 2mm, 1cm or 2cm markers in the small intestine. The fluid and 2mm markers reached the end of the small intestine within 2 hours. Kass et al. (1980) reported small intestinal transit rates of 2.2-3.4 hours in adult pigs. It appears therefore, that the passage of digesta through the small intestine is similar for humans and pigs.

Table 1.5. The secretion rate of some of the components of bile in the adult human (65kg) and the growing pig (25kg).

Component (unit/kgBW/kgDM) [*1]	Human [*2]	Pig [*3]
Secretion rate (ml)	23.1	22.5
Mucin and pigment (mg)	9-30	6
Acids (g)	4.4-6.8	0.2-1.0
Lipids (g)	0.5-4.2	0.4
Fatty acids (mg)	85	85
Solids (g)	0.6	2.4
Phosphorus (mg)	6-35.5	5
Choline (mg)	13-115	20
Cholesterol (mg)	10-30	10
Neutral fat (mg)	50	45-100
Protein (mg)	45-70	95

[*1] All components in the table are expressed as ml, mg, or g/kg body weight/kg dry-matter eaten.

[*2] Bockus, 1976a; Davenport, 1982; Smith et al., 1983; Geigy Scientific Tables, 1970.

[*3] Pond and Houpt, 1978.

1.4.3. THE LARGE INTESTINE.

The large intestine consists of the caecum, ascending, transverse, descending and sigmoid colon, rectum and anal canal. The lengths of the large intestine of the pig and adult human are given in Table 1.1, and pHs in Table 1.4.

The main function of the large intestine is for water and electrolyte absorption, but it may also be important for the metabolism of N entering from the small intestine. It differs from the small intestine in that it has no permanent folds of the mucosa and sub mucosa except in the rectum and anal canal, and it has no villi. The caecum in humans has a short (about 9cm), narrow (0.5cm) blind-ended tube opening from it called the vermiform appendix, which is absent in the pig, although in humans it appears to have no digestive function (McMinn and Hobdell, 1974).

The rate of passage of digesta through the large intestine is much slower than through the rest of the gut (Pond and Houpt, 1978). In humans, the time taken for a meal to pass through the entire gut varies considerably from person to person, and according to the diet eaten (it may take from 3-4 hours to 3-4 days), (Bockus, 1976c), with the average time being about 2.5 days (Magee and Dalley, 1986). Therefore the actual time the digesta spends in the large intestine is about 2 days. In a study with growing pigs (Seerley et al., 1962), transit rates of approximately 2-4 days were reported, by measuring the recovery of 95% of an ingested marker in the faeces. Clemens and Stevens (1980) estimated that fluid markers moved through the large intestine at a rate of 30-40cm/h, and particulate markers 16-19cm/h. The time taken to recover 85% of the markers in the faeces ranged from 43h for fluid markers to over 240h for 2cm markers. The time that digesta spends in the large intestine of the pig appears to be 1-3 days.

Another feature of the large intestine is that it hosts a vast microbial population, in comparison with the rest of the gut.

1.4.4. THE GUT MICROFLORA.

The stomach and small intestine in humans have traditionally been considered to be sterile (Hill, 1982; Ratcliffe, 1985), and Kalser et al. (1966) reported that under normal conditions in humans, the growth of bacteria in the small intestine is limited, and the concentrations low, although none of the ileal aspirates examined were sterile. Many other studies have also given conflicting results as to whether the upper digestive tract is colonised with microorganisms or not. It is likely that there are some microorganisms present, although in low numbers.

It has been reported that there are normally less than 10^3-10^5 organisms/ ml of digesta in the jejunum, and these are mainly of the oropharyngeal type (Dellipiani and Girdwood, 1964; Goldstein et al., 1961). However, Hungate (1978) has shown that a 10 minute exposure of saliva to an aqueous solution of hydrochloric acid (pH 2) as is found in the stomach, kills all bacteria except a few sporeformers, suggesting that most of the digesta entering the small intestine from the stomach should be free from bacteria.

Hungate (1978) reported that there were no microbial colonies in the human stomach or duodenal digesta, but found 1.3×10^3 anaerobes/ml of digesta in the jejunum and less than 10 aerobes/ml in one subject, and in another subject 2.8×10^3 anaerobes/ml and 40 aerobes/ml. The main species present were micrococcus, streptococcus, lactobacillus, veillonella, haemophilus, and clostridium. Gracey (1981) has reported that the upper jejunal secretion of a healthy adult human has less than 10^4 bacteria/ml, except after meals when significant numbers of bacteria may enter the small intestine with the food. Other workers have shown that jejunal samples taken after a fasting period, are sterile in normal individuals, and the presence of faecal-type organisms in the upper small intestine was abnormal (Tabaqchali et al., 1966). Colonic type bacteria may exist in the

ileum (Kalser et al., 1966), although the reported data concerning the microbial colonisation of the human upper digestive tract are conflicting. The differences reported may exist largely due to the difficulty in obtaining accurate measurements because of the limitations in techniques available for this type of work, especially in early studies. However, it seems that there is a small degree of colonisation, increasing in more distal parts of the small intestine, and that numbers of bacteria enter with the food having avoided destruction in the stomach.

The human colon displays a flora which is complex and distinct for each individual. Croucher et al. (1983), using 4 sudden death victims, have shown that there are more anaerobes than aerobes in the human colon, with approximately $1.5x10^6$-$2.7x10^8$ anaerobes/g colon wall and $3.8x10^3$-$3.9x10^7$ aerobes/g colon wall. The major species of anaerobes were bacteroides and fusobacterium.

Moore et al.(1978) have stated that each animal species appears to have a different bacterial population. They have shown that rapid growth of bacteria occurs in the human caecum and ascending colon after which the total number and relative numbers of each type remain constant. Savage (1983) has also reported that the composition of the microflora found in the human colon is relatively constant and independant of the diet.

Studies in the pig, measuring organic acid production have suggested that bacteria are present in the stomach (Cranwell, 1968), at high levels and are found right through the gut throughout life (Pond and Houpt, 1978). Although bacteria are found in the stomach of pigs, the microbial activity here is far lower than that found in other parts of the gut. The microbial population appears to be fairly stable in the pig gut, with the total aerobic, anaerobic and lactobacilli counts remaining reasonably stable regardless of the diet (Willingale and Briggs, 1955; Larson and Hill, 1955). The main strains of microorganisms found in the stomach and upper small

intestine of the pig are lactobacilli, streptococci, E.coli, veillonellae and yeasts (Fuller et al., 1978; Barrow et al., 1980), which is similar to the types of bacteria found in humans.

The predominant strains of bacteria found in the large intestine of the pig are bacteroides and lactobacilli, which is also similar to humans. The total numbers of aerobes has been reported to be $2.5x10^8$-$1.0x10^9$/g dry-matter, and anaerobes to be $1.0x10^9$-$2.5x10^9$/g dry-matter in the large intestinal contents of growing pigs (Wilbur et al., 1960), which again is similar to the numbers found in the human large intestine.

The main strains of bacteria found in pig faeces are coliforms (Horvath et al., 1958) and the same genera of anaerobic microorganisms are commonly found in the faeces of humans and the pig (Raibaud et al., 1982). A summary of the gut microflora, comparing the two species is given in Table 1.6.

The upper tract of pigs may be host to a larger microbial population than humans, due to the environment in which pigs are reared and subsequently housed. The pig's environment is not always as hygenic as that of humans, with the pig eating and defaecating in close proximity, as well as the pig walking in faeces and then standing in its food. In addition to this, the food eaten by pigs may be more contaminated by bacteria than human food as may be the eating conditions. If pigs were reared under conditions similar to those of humans, the upper tract may not become colonised to the same extent and the two species may be more similar in respect to the microflora.

The gut microflora are able to degrade amino acids by deamination, decarboxylation and other reactions, described in Section 1.2.3 of this thesis. The bacteria most often found in the upper tract of both species are the lactobacilli and streptococci, which appear to have limited ability to deaminate or decarboxylate amino acids

Table 1.6. The main species of microorganisms (log_{10} viable count/g contents) found in various regions of the digestive tract of the adult human (65kg) and the growing pig (25kg).

	Site[*1]						
Strain	S	SI1	SI2	SI3	SI4	LI	F
Human[*2]							
E.coli	N[*3]	N	N	N	5.6	6.5	7.6
Clostridia	N	N	N	+[*3]	+	2.0	4.0
Streptococci	N	N	N	+	+	2.6	7.0
Lactobacilli	1.0	2.0	2.0	3.5	4.2	6.4	6.4
Yeasts	1.0	1.0	1.0	1.0	1.0	2.0	1.0
Bacteroides	N	N	N	N	5.2	7.9	10.0
Veillonellae	N	N	N	+	+	+	
Pig[*4]							
E.coli	4.0	3.4	3.7	4.5	5.3	6.5	6.8
Clostridia	1.2	N	N	1.7	3.0	+	N
Streptococci	5.2	4.2	4.5	6.0	6.5	7.0	7.2
Lactobacilli	7.8	6.5	7.0	7.6	8.0	8.6	8.9
Yeasts	4.3	3.9	3.9	4.4	4.0	4.0	4.2
Bacteroides	N	N	N	N	N	7.4	7.6
Veillonellae	5.5	3.7	4.5	3.9	3.9	7.0	

[*1] S = stomach, SI = small intestine (in four sections 1,2,3 and 4), LI = large intestine, F = faeces.

[*2] Williams Smith, 1965; Drasar et al., 1969; Drasar and Hill, 1974; Moore et al., 1978; Drasar and Barrow, 1985.

[*3] N = not present, + = present in small numbers only.

[*4] Horvath et al., 1958; Williams Smith, 1965.

(Drasar and Hill, 1974). E.coli found in the upper tract of pigs in smaller numbers, and sometimes found in humans, are able to deaminate several amino acids, including the essential amino acids: histidine, cysteine, tyrosine, threonine and tryptophan. Some bacteria are also able to decarboxylate lysine, arginine, glutamate and proline, while other strains of bacteria can decarboxylate histidine, tyrosine and leucine as well (Drasar and Hill, 1974). Some strains of bacteria found in the gut may also possess the ability to degrade some amino acids. This may have an effect on the determination of amino acid digestibility at the ileum, especially when using the ileostomate, whose terminal ileum may have 100 times the density of microorganisms compared to the normal ileum (Wrong et al., 1981). The main types of bacteria found in ileostomy output, however, possess limited ability to hydrolyse protein and ferment amino acids (Drasar and Hill, 1974). Ratcliffe (1985), referring to the work by Salter (1984) with germ-free and conventional animals, stated that the effect of enteric bacteria on protein digestion in the small intestine of the pig is likely to be small.

To conclude it appears that the types of microorganisms found throughout the gut are similar in humans and the pig (Drasar and Barrow, 1985). However, there appear to be differences in the numbers present, with the pig's upper tract hosting a greater microbial population than that of the human. The data in the area of gut microbiology must be interpreted with caution, as the techniques used in early work have been greatly improved over the years. Even modern techniques for sampling and detection are not perfect, sometimes leading to contamination and/or incomplete detection of organisms (Drasar and Barrow, 1985).

1.4.5. COMPARATIVE DIGESTIBILITY STUDIES.

When evaluating the use of the growing pig (25kg) as a model for digestibility studies in humans, direct comparisons between the species need to be made to determine the extent of digestion and absorption, and therefore to establish just how closely the pig resembles humans. Few digestibility studies involving a direct comparison between pigs and humans have been reported. In a study by Forsum et al. (1981), true faecal protein digestibilities were compared between adult humans, the growing pig (12kg) and the rat consuming similar diets. The results were 84.3, 87.6, and 86.1% for the human, pig and rat respectively. The endogenous faecal N levels were calculated using different techniques for each of the three species. The results were similar for all three species. However, it was suggested that the pig would serve as a better model for man than the rat when testing human foods, because if the protein quality is poor there may be decreased diet consumption when using rats. Jones (1980) suggested that the pig would be a more appropriate animal model than the rat for studies involving the effects of dietary fibre on both the small and large intestine. Nyman et al. (1986) have also suggested that larger animals such as the pig would be more suitable, because smaller animals consume more energy/unit body weight.

The study by Forsum et al. (1981) also reported similar values for digestible energy, measured on the faeces, for two different diets, for the pig and humans. These workers suggested that further studies should use the pig as an experimental model for man in the evaluation of human diets. It appears, therefore, that the pig closely resembles humans for the digestibility of protein and energy. The pig has also been suggested to be a suitable model for humans for fibre digestion (Jones, 1980; Fleming and Wasilewski, 1984; Low, 1985).

As yet, a comprehensive comparison of protein and amino acid digestibilities determined at the end of the small intestine or in the faeces of these two species, has not been undertaken.

It is concluded, however, that the main differences between the two species is the relative organ sizes, with the pig having a considerably larger tract per unit body weight. However, it must be pointed out that the 25kg pig is a growing animal, which has been compared to an adult human, therefore it would be expected that when expressed on a body weight basis, the organ sizes will be greater for the growing animal. When expressed on the basis of food intake, the values for the adult human and growing pig are very similar (Moughan and Rowan, 1989). Also when interpreting data on organ sizes, it must be remembered that measurements taken post mortem will be greater for length and diameter, than if they were taken from a live subject, so much variation exists in the reported data.

The gut secretions appear to be similar in composition and rate of excretion when related to body weight and dry-matter intake, in the two species, although the 25kg pig may have a greater daily dry-matter intake than the average adult human. Therefore, the total amount of some of the components of the gastric juice, pancreatic and bile secretions may be greater for the pig than for the adult human. When the amount of protein secreted in the pancreatic juice is expressed as a daily rate, the amounts are very similar for the two species. The digestive enzymes are also similar with regards to the site of activity in the gut and substrate specificity. The transit rates through the gut also appear to be similar, although gastric emptying may be somewhat slower in the pig than humans, and the time spent in the large intestine may be greater for the pig. Overall, however, and with respect to the anatomy, physiology, microbiology, and biochemistry of the digestive tract, the growing pig appears to be a suitable choice of model animal for the adult human.

1.5. OVERALL CONCLUSION.

Based on the above review two major (1-2) and several minor (3-7) research aims were established:

1. To compare the ileal and faecal digestibility of protein and amino acids in humans fed the same diet, using the ileostomate for collection of ileal digesta.

2. To evaluate the growing pig as a model animal for humans for determining the ileal and faecal digestibility of amino acids from human-type diets.

3. To establish appropriate conditions for the analysis of amino acids including tryptophan, in diet, ileal digesta and faeces samples.

4. To evaluate the use of Celite as a non-digestible marker in studies involving humans.

5. To determine the ileal endogenous amino acid losses in humans and pigs following a protein-free meal, to allow calculation of true digestibilities.

6. To determine the levels of bacteria present in digesta and faeces samples by measuring bacterial marker compounds, and to determine the extent of bacterial activity in these samples by measuring the extent of fibre digestion, to evaluate the suitability of the ileostomate as a model for intact subjects.

CHAPTER 2.

INVESTIGATION OF HYDROLYSIS CONDITIONS FOR THE DETERMINATION OF AMINO ACIDS IN BIOLOGICAL SAMPLES.

GENERAL INTRODUCTION.

In a study to determine the digestibility of amino acids, the analysis of amino acids is of central importance. The chemical analysis of samples for the determination of amino acids involves hydrolysis of the protein to release the constituent amino acids, which can then by separated by various chromatographic techniques to allow quantification of the amino acids present. The most commonly used technique is the classical automated amino acid analysis based on ion-exchange separation and detection after post-column derivatisation with ninhydrin (Moore and Stein, 1951). Subsequent developments have improved the sensitivity of analyses, and the speed at which they are performed. A further improvement in sensitivity can be achieved by using fluorescent detection methods, by forming a fluorescent derivative of the separate amino acids. However, secondary amino acids such as proline can not be detected by these methods (Hirs and Timasheff, 1978). Other reagents have also been used for forming derivatives to allow detection of the amino acids (Ashman and Bosserhoff, 1984).

In recent years, alternative chromatography techniques have been developed and applied to the separation of amino acids, to decrease the time required for separation and increase the sensitivity. The use of reverse-phase high performance liquid chromatography (HPLC) for separation is a rapid method, and is becoming more popular due to the flexibility of the equipment for application to separation of other substances (Hancock and Harding, 1985; Danakov, 1985). HPLC has been applied to acid hydrolysates of foods (Hurst and Murtin, 1980; Kan and Shipe, 1981), and the separation of most amino acids was achieved. There do not appear to be any problems due to the

possible presence of interfering substances after hydrolysis of non-pure protein samples. Kan and Shipe (1981) compared HPLC with ion-exchange separation and reported similar amino acid values for both methods for a variety of food protein hydrolysates. Hill et al. (1979) have shown that HPLC may not offer the same extent of specificity as traditional methods, and Kan and Shipe (1981) found that threonine and glycine coeluted for their hydrolysates with the conditions they applied. Ligand-exchange chromatography is also used for the separation and isolation of compounds capable of forming complexes with metal ions bonded to a stationary phase, and allows for the separation of quite similar compounds (Danakov, 1985). This method does not appear to be used for routine analyses as yet. Gas-liquid chromatography (GLC) can be used for the separation of amino acids, and provides a sensitive, accurate and rapid means of analysis (Blackburn, 1978). Several studies (Gehrke and Leimer, 1971; Cliffe et al., 1973; Kirkman, 1974) have shown the analysis of biological samples by GLC to be comparable to results obtained by ion-exchange separation. In spite of these points, GLC is not commonly used for the analysis of amino acids, probably because there are some problems with the preparation of stable volatile amino acid derivatives (Blackburn, 1978).

Post-column derivatisation using ninhydrin or the formation of fluorescent derivatives is commonly used with ion-exchange chromatography (Blackburn, 1978). The major disadvantage is the resultant band spreading (Hancock and Harding, 1985), and sometimes some amino acids can not be detected. Pre-column derivatisation may allow for better resolution of peaks (Ashman and Bosserhoff, 1985), which will be increasingly important as the time required for the separation of a complex mixture of amino acids is shortened. Pre-column derivatisation must occur to a high degree, as the presence of side products will cause difficulty in separation and quantification of the amino acid derivatives.

Overall, it appears that the standard methods of ion-exchange separation and post-column derivatisation are still acceptable, as

the only real disadvantages are longer time, and some possible lack of sensitivity. The standard method of acid hydrolysis, however, does not give quantitative yields of all amino acids, with tryptophan usually being completely destroyed. Tryptophan can, however, be measured after alkaline hydrolysis. In addition to tryptophan destruction, methionine and cysteine may also be oxidised during acid hydrolysis, and are usually measured after acid hydrolysis following performic oxidation. Moreover, materials as physically diverse as food, faeces and digesta may require different hydrolysis conditions to give optimal amino acid yields. The studies described in this section aimed to evaluate several aspects of acid and alkaline hydrolysis procedures and to determine optimal hydrolysis conditions for food, faeces and digesta samples for all the common amino acids.

SECTION 2.1. ACID HYDROLYSIS FOR AMINO ACID ANALYSIS.

Methods of protein hydrolysis using acid have been studied and reviewed by several workers (Davies and Thomas, 1973; Bech-Andersen et al., 1979a; b; 1980; Rudemo et al., 1979; 1980; Mason et al., 1979; 1980). The most widely accepted method uses 6M hydrochloric acid (HCl), (Blackburn, 1968; 1978). The material is hydrolysed in vacuo at 110°C for 24h. Davies and Thomas (1973) reported that 6M HCl in comparison with other acids gave the highest yield for most amino acids in food proteins. This method, however, does not lead to quantitative measurement of some amino acids from the protein, and the time of hydrolysis required to give optimal values for amino acids may depend on the nature of the sample being hydrolysed (Kohler and Palter, 1967; Garnett, 1985). The following study consisted of two parts. The first compared two methods for the hydrolysis of samples in acid (sealed tubes and round-bottomed flasks) for amino acid yield, including the yields of methionine and cysteine following performic oxidation of the samples. The second part investigated the hydrolysis time required to give optimal yields of amino acids, and the use of correction factors when using one hydrolysis time only.

PART 1. THE USE OF SEALED TUBES VERSUS ROUND-BOTTOMED FLASKS FOR ACID HYDROLYSIS OF BIOLOGICAL SAMPLES.

a. INTRODUCTION.

Oxidation of methionine to methionine sulphoxide then methionine sulphone, and of cystine and cysteine to cysteic acid occur incompletely during acid hydrolysis, so for quantitation of these amino acids, the sample is oxidised before it is hydrolysed. Performic acid is usually used as the oxidising agent, and the sample is then hydrolysed in 6M HCl, with the sulphur amino acids being detected as methionine sulphone and cysteic acid (Moore, 1963). The oxidised forms of these amino acids are relatively stable

to acid hydrolysis (Schram et al., 1954; Kohler and Palter, 1967; Davies and Thomas, 1973; Blackburn, 1978). Tyrosine is usually destroyed by this procedure, especially if halide ions are present, however the presence of phenol in the oxidation mixture appears to prevent this (Andrews and Baldar, 1986). It is usually necessary to carry out acid hydrolysis of both the oxidised and the non-oxidised sample to determine all amino acids accurately, and to ensure that no other substances elute in the same position as methionine sulphone and cysteic acid during analysis of the amino acids. For example, O-phosphoserine is known to elute in the same position as cysteic acid but this is usually destroyed in HCl (Glazer et al., 1976).

For the acid hydrolysis of non-pure protein samples, it has been found that a higher humin concentration is formed with small acid to sample ratios (eg. 10mg/ml), leading to variable losses of threonine, serine and cysteine (Schram, et al., 1953). A higher acid to sample ratio (eg. 2.0ml/mg) has been shown to give better results for feedstuffs (Booth, 1971; Blackburn, 1978), containing high levels of carbohydrate. A higher acid to sample ratio can be achieved by using round-bottomed flasks with hydrolysis under reflux (Davies and Thomas, 1973; Mason et al., 1980; Andrews and Baldar, 1986). This method (Mason et al., 1980) can be modified to involve oxidation of feedstuffs with performic acid in the presence of phenol, followed by hydrolysis under reflux. Mason et al. (1980) reported good recovery of all amino acids in feedstuffs, except for tyrosine and tryptophan.

In this study, a comparison was made between the round-bottomed flask method (Andrews and Baldar, 1986) which provides a greater acid to sample ratio, and the traditional sealed, evacuated tube method for hydrolysis of both performic acid oxidised and non-oxidised samples.

B. METHODS AND MATERIALS.

REAGENTS.

All reagents used were of AnalaR quality.
Hydrochloric acid (HCl), distilled, BDH, England.
Hydrogen peroxide (H_2O_2), Ajax Chemicals, Australia.
Formic acid (99%), Ajax Chemicals, Australia.
Hydrobromic acid (HBr), May and Baker Ltd., England.
Phenol (crystalline), BDH, England.
Sodium metabisulphite, Selby-Wiltons Scientific Ltd.,N.Z.
Methionine sulphone, Cysteic acid, Sigma Chemical Co., U.S.A.
BCM (Beckman calibration mixture), Beckman, U.S.A.

CHEMICAL ANALYSIS.

I. Round-bottomed flask method of hydrolysis (Andrews and Baldar, 1986):

i. 6M HCl hydrolysis: Duplicate 50mg samples of a freeze-dried and finely ground (1mm) diet (based on meat/vegetables/cereal/dairy products), were hydrolysed with 50ml of 6M HCl containing 50mg of phenol, under reflux for 24h.

ii. Performic acid oxidation: Duplicate 50mg samples of the diet were placed into 250ml round-bottomed flasks. The oxidation mixture, which consisted of 0.5ml 30% H_2O_2 in 4.5ml 88% formic acid, was incubated at 30°C for 1h, then cooled in an ice bath for 15 minutes. All of the oxidation mixture was added to the sample (i.e 5ml/duplicate), and the oxidation proceeded at 0-4°C for 16h. Sodium metabisulphite (approximately 0.84g) was added to degrade the excess reagent. Hydrolysis was then carried out under reflux as described above.

II. Sealed, evacuated tube method of hydrolysis (Glazer et al., 1976):

i. 6M HCl hydrolysis: Duplicate 50mg samples of the diet were mixed with 10ml of 6M HCl (containing 0.5mg phenol/ml) in large (30ml) glass hydrolysis tubes. The mixture was frozen before the tube was evacuated to remove all traces of oxygen, and the tube was sealed under vacuum. Hydrolysis was carried out at 110°C in an oven for 24h.

ii. Performic acid oxidation: Performic acid was prepared by allowing a mixture of 1.0ml of 30% H_2O_2 and 9.0ml of formic acid to stand at room temperature for 1h, and was then cooled to 0-4°C. Two ml of the oxidation mixture were added to duplicate 50mg samples of the diet. The oxidation was allowed to proceed at 0°C for 4h after which time the performic acid was destroyed by adding 0.15ml of cold 48% (w/v) HBr per ml of performic acid used. The bromine and formic acid were removed by evaporation under high vacuum. Hydrolysis was then carried out using 10ml 6M HCl (plus phenol) as described above.

After hydrolysis, all hydrolysates were quantitatively transferred to 100ml volumetric flasks, and made up to volume with deionised water. The hydrolysates were filtered with the first 3-5ml discarded, then 2ml were taken from each filtrate and evaporated to dryness under vacuum, and the dried samples were stored at -20°C until being analysed. Loading buffer was added to each dried sample just prior to loading onto a Beckman 119 BL amino acid analyser, which had been calibrated with a 25nmol BCM. The analyser had a three buffer system (Na^+ citrate), with detection based on the ninhydrin reaction, and the results were obtained using a Spectrophysics SP4290 integrator (Watson Victor Ltd., N.Z.). The positions of elution of all amino acids are illustrated in Fig. 2.1.

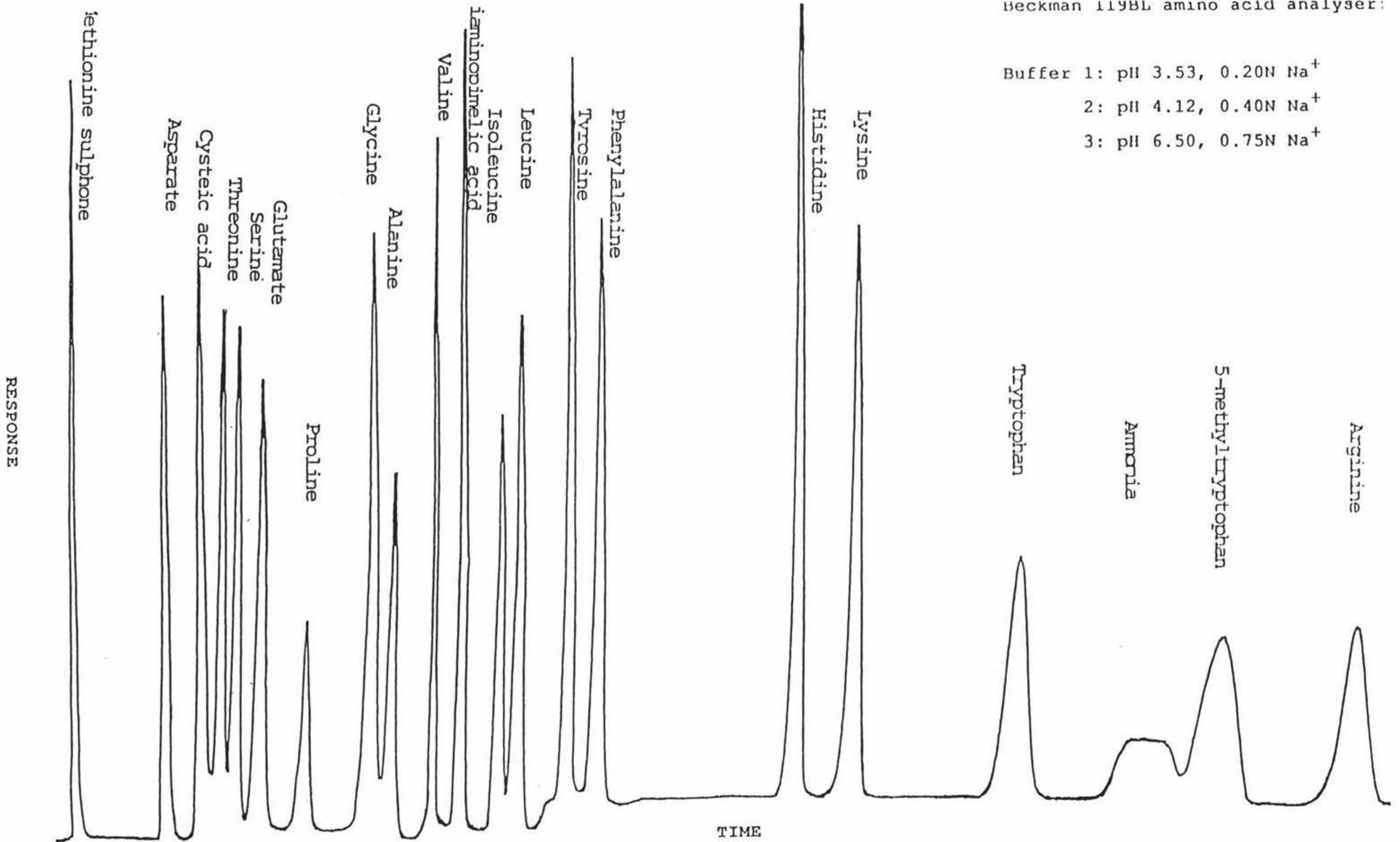


Figure 2.1. A chromatogram of a mixture of amino acids including methionine sulphone, cysteic acid, diaminopimelic acid, tryptophan and 5-methyltryptophan.

c. RESULTS AND DISCUSSION.

The mean values for each amino acid are given for each of the four methods in Table 2.1. There was generally good agreement between duplicates (less than 5% difference of the mean value), although the agreement between duplicates after acid hydrolysis in round-bottomed flasks was often poor, with the difference being up to 15% units for serine, and 5-10% units for many of the other amino acids. This may have been due to the presence of oxygen during hydrolysis.

The sealed tube method for acid hydrolysis gave higher values for many of the amino acids than the round-bottomed flask method, although the differences were often small. In view of this, and taking into account the better agreement between duplicates, the sealed tube method was used for hydrolysing non-pure protein samples in this study.

In contrast, the round-bottomed flask method for hydrolysis following performic oxidation gave significantly greater methionine sulphone and cysteic acid values than the sealed tube performic oxidation method. It was also noted that the amino acid values after performic oxidation and hydrolysis in the round-bottomed flask were very similar to the values obtained for amino acids (except methionine and cysteine) after acid hydrolysis of the non-oxidised samples in sealed tubes. On average, the amino acid values were the same if not slightly higher after performic oxidation although the agreement between duplicates was poorer than for the sealed tube method. Mason et al. (1979) have shown that all but a few of the common amino acids can be accurately determined after hydrolysis of oxidised samples using round-bottomed flasks. The method allows the oxidation and hydrolysis of the sample to be carried out in the same vessel, so minimising errors and losses, and the results given are said to be reproducible (Andrews and Baldar, 1986). The amino acids which were lower after performic oxidation and hydrolysis in round-bottomed flasks were aspartate, threonine, serine, glutamate, and

Table 2.1. Comparison of mean amino acid levels*1 (nmol/mg freeze-dried sample) in a diet sample determined after hydrolysis in sealed tubes or round-bottomed flasks with or without prior oxidation with performic acid.

	METHOD			
	SEALED TUBE		ROUND-BOTTOMED FLASK	
Amino acid	oxidised	non-oxidised	oxidised	non-oxidised
Lysine	90.0	82.4	84.3	81.8
Histidine	23.3	35.2	34.9	32.8
Arginine	67.2	77.0	78.6	83.2
Aspartate	144.5	156.2	149.0	131.3
Threonine	69.9	73.5	65.4	60.0
Serine	87.9	92.7	78.9	82.4
Glutamate	262.7	280.9	239.8	280.5
Proline	94.1	124.4	96.6	129.8
Glycine	117.0	121.0	122.3	131.7
Alanine	103.0	111.9	114.0	117.0
Valine	88.9	92.5	102.6	84.5
Isoleucine	63.1	67.2	73.1	67.7
Leucine	117.9	125.5	130.6	129.9
Tyrosine	3.6	46.1	47.5	47.9
Phenylalanine	60.1	62.7	65.0	56.2
Methionine*2	28.8	28.5	44.7	27.9
Cysteine*2	19.5	15.1	34.2	0.0

*1 mean of duplicates.

*2 Methionine determined as methionine, and cysteine as cysteine after hydrolysis of a non-oxidised sample, or methionine as methionine-sulphone, and cysteine as cysteic acid after hydrolysis of an oxidised sample.

proline, which were found to be lower by 5, 12, 16, 16, and 25% respectively than the samples hydrolysed in sealed tubes. Efforts, however, to try and improve the yields of these amino acids by the round-bottomed flask method failed. The lower yields are probably due to oxidation of the amino acids under these hydrolysis conditions.

It is concluded, therefore, that the analysis of samples for all amino acids except methionine, cysteine and tryptophan is best made after acid hydrolysis in sealed, evacuated tubes. In addition, methionine and cysteine should be determined as methionine sulphone and cysteic acid after performic acid oxidation of the sample followed by acid hydrolysis in round-bottomed flasks.

PART 2. INVESTIGATION OF HYDROLYSIS TIME FOR THE DETERMINATION OF AMINO ACIDS IN DIVERSE SAMPLES.

a. INTRODUCTION.

Some amino acids are often partially lost during acid hydrolysis, for example tryptophan, tyrosine, serine and threonine (Rees, 1946). The destruction of serine during acid hydrolysis is usually constant for all proteins, but threonine and tyrosine destruction is often variable for different proteins (Glazer et al., 1976). As well as losses of several amino acids, longer hydrolysis times are required to give quantitative evaluation of some amino acids. For example, valine, leucine and isoleucine are often released more slowly than other amino acids during acid hydrolysis (Blackburn, 1968). Glutamine and asparagine cannot be determined after acid hydrolysis as the amide group is hydrolysed to form glutamate and aspartate respectively.

The most accurate method for analysing amino acids which are either not fully released or are degraded, is to carry out the hydrolysis over several different periods of time, and to extrapolate to give their correct values (Kohler and Palter, 1967; Tkachuk and Irvine, 1969; Davies and Thomas, 1973). Also, the inclusion of phenol in the hydrolysis mixture offers some protection against destruction, especially for tyrosine (Glazer et al., 1976).

As discussed above, the standard 24h hydrolysis time does not give quantitative yields for all amino acids, and different types of samples may be hydrolysed to different extents after 24h. If different types of samples are to be compared with each other, therefore, as in digestibility studies, it is important to determine if the samples behave similarly under acid hydrolysis. In digestibility studies, digestibility coefficients are calculated using amino acid values determined on a diet sample and on either ileal digesta or faecal samples. Therefore, it needs to be

established whether the digesta and faecal samples undergo hydrolysis, with subsequent release of amino acids, in a similar manner to the diet sample. The study reported in this section investigated the effect of hydrolysis time on amino acid yield for different types of samples, in particular a diet sample, ileal digesta sample and faecal sample. If for some amino acids the change over time was not similar between the diet and either ileal digesta or faecal samples, then correction factors were derived to allow prediction of the level of the amino acid at a set time.

b. METHODS AND MATERIALS.

The reagents used are the same as those described in Part 1.

CHEMICAL ANALYSIS.

Single samples of diet, ileal digesta and faeces were hydrolysed in sealed evacuated tubes, for analysis of all amino acids except tryptophan, methionine and cysteine. These samples were collected from subjects as described in a subsequent chapter (Section 4.2), and were freeze-dried and finely ground (1mm). The diet consisted of a meat/vegetable/fruit/cereal/dairy products base, and the ileal digesta and faeces samples had been collected from intact and ileostomised animals which had been eating this diet. Duplicate 50mg samples of each material were weighed into large hydrolysis tubes as described previously (Part 1, method II.i) and hydrolysed for 16, 24, 48 or 72h. The hydrolysates were processed and analysed as described previously (Part 1).

DATA ANALYSIS.

The data for amino acid yield after different times of hydrolysis were analysed statistically to determine whether there was an effect of time on amino acid yield and whether this effect differed by source of material (interaction).

A linear model which included terms for source, a linear effect of time, departure from linearity of the time effect (lack-of-fit), linear time*source and departure from linearity*source was fitted to the data and reductions in sums of squares were used to determine levels of significance. There was not a true statistical replication (i.e. different randomly-chosen samples) at each time for each source of material, but rather duplicate observations on the same sample. Because of this and the high degree of precision of amino acid analysis, the error sums of squares obtained after fitting the

model, was very low.

If there was a significant effect of time on amino acid yield and given that the amount of material remaining unhydrolysed decreases with time of hydrolysis, it would be expected that the form of response would be curvilinear rather than linear. Moreover, if all the interactions occuring during hydrolysis were fully understood, then for those amino acids showing a significant decrease or increase in yield after 16h hydrolysis, the mean determined values would be expected to fit the defined mathematical response model perfectly. In the present situation, however, such a model is not defined, nor is it appropriate to examine the statistical fits of a selection of possible different response models. In the present study, and where there was a significant curvilinear effect and interaction, the approach of determining correction factors (i.e. the amino acid yield at the time giving the maximum value expressed as a proportion of the yield at 24h) was adopted. The latter factors may be used to correct a determined amino acid yield after a single 24h hydrolysis, and their calculation requires no assumptions to be made about the shape of the relationship between yield and hydrolysis time.

c. RESULTS AND DISCUSSION.

The hydrolysis times chosen for this type of study should be well-spaced over a wide range, and include a lower time when amino acid loss would be expected to be minimal and an upper time whereby hydrolysis would be expected to be complete. The times adopted in the present study (16, 24, 48 and 72 h) have commonly been used in past studies. It is expected that by 72h, hydrolysis will be complete (Mahowald et al., 1962; Noltmann et al., 1962; Kohler and Palter, 1967; Tkachuk and Irvine, 1969).

In the present study, the difference between the duplicates was generally less than 5% units (difference expressed as a proportion of the mean value), and this level of agreement was similar across the three sources. The mean raw data are given in Appendix 1. The amino acids which did not show a curvilinear response over time were tyrosine, valine, arginine, glycine and leucine. For valine and tyrosine there was no interaction between samples, and these amino acids, therefore, do not require correction for loss or gain after hydrolysis when calculating digestibility coefficients. The tyrosine values did not change with time while the valine values increased linearly with time. When absolute values for valine are required correction to 72h should be made, using regression equations (Table 2.2). For arginine, glycine and leucine the change over time was not proportional between samples. However, regression equations were derived for these amino acids for each sample (Table 2.2). A positive slope indicates an increase with time while a negative slope indicates a decrease. For some of these amino acids in some samples, the coefficients of determination (R^2) were very low, for example glycine in the diet sample and arginine and glycine in the faeces sample. This indicates that the data were spread widely around the line of best fit for those amino acids.

Table 2.2. Slopes and intercepts for linear regressions of amino acid content on hydrolysis time for diet, ileal digesta and faeces samples.

Sample/ Amino acid	Slope	(± S.E.)	Y-intercept	(± S.E.)	R^2
Diet					
Arginine	0.061	(0.044)	64.40	(2.01)	0.49
Glycine	0.030	(0.107)	90.07	(4.90)	0.04
Leucine	0.233	(0.072)	122.38	(3.29)	0.84
Valine	0.328	(0.010)	73.66	(0.47)	1.00
Ileal digesta					
Arginine	0.170	(0.017)	32.57	(0.78)	0.98
Glycine	0.628	(0.155)	93.00	(7.07)	0.89
Leucine	0.406	(0.190)	53.97	(8.68)	0.69
Valine	0.402	(0.112)	46.31	(5.10)	0.87
Faeces					
Arginine	-0.090	(0.008)	86.34	(3.11)	0.47
Glycine	0.158	(0.104)	215.63	(4.76)	0.53
Leucine	-0.078	(0.148)	172.28	(6.75)	0.12
Valine	0.281	(0.071)	142.95	(3.23)	0.89

The amino acids for which there was a curvilinear effect and an interaction between samples (significant at P<0.05) were phenylalanine, histidine, aspartate, glutamate, proline, alanine and threonine. These amino acids require correction to give the correct values for calculating digestibility coefficients. The correction factors for these amino acids are given in Table 2.3. For some of these amino acids in some samples the maximum value was obtained after the 24h hydrolysis, and so the correction factors were 1.00. The amino acids which tended to increase over time were phenylalanine, glutamate and alanine, while histidine, aspartate, proline and threonine tended to decrease. The degree of over or underestimation varied with the amino acid. The absolute error for glutamate in ileal digesta, for example, when measured after 24h hydrolysis rather than 72h, is 22nmol/mg of freeze-dried sample. Whereas for proline in ileal digesta, when measured after 24h rather than 72h, the difference was only 2nmol/mg freeze-dried sample.

The amino acids for which there was a curvilinear response but no significant interaction between sources, were lysine, serine and isoleucine. These amino acids do not require correction if the values are to be used to calculate digestibility coefficients. However, if absolute amino acids values are required for any one sample, correction may need to be made if the 24h value is not the maximum. The serine values decreased with time while the lysine and isoleucine values increased. Correction factors have been determined for these amino acids and are given in Table 2.4.

In several studies investigating the hydrolysis of foods (Kohler and Palter, 1967; Tkachuk and Irvine, 1969; Mawer and Nixon, 1969; Davies and Thomas, 1973; Garnett, 1985) a number of hydrolysis intervals have been used. All of these studies have shown that serine and threonine values decrease with hydrolysis time, and valine and isoleucine increase with time. The shapes of the curves reported by Davies and Thomas (1973) were generally similar to those found for the presently reported study (see Appendix 1). The curves

Table 2.3. Correction factors[*1] for the amino acids for which there was a curvilinear effect of time and showing a significant interaction (sample type*time) after hydrolysis of a diet, ileal digesta and faeces sample for 16, 24, 48 or 72h.

	Correction factor		
Amino acid	Diet	Ileal digesta	Faeces
Glutamate	1.11	1.20	1.00
Phenylalanine	1.17	1.18	1.03
Threonine	1.03	1.09	1.11
Alanine	1.05	1.16	1.00
Aspartate	1.02	1.03	1.00
Histidine	1.09	1.19	1.00
Proline	1.05	1.02	1.00

[*1] Correction factors determined by expressing the maximum amino acid yield as a proportion of the yield at 24h.

Table 2.4. Correction factors[*1] for the amino acids for which there was a curvilinear effect of time and not showing a significant interaction (sample type*time) after hydrolysis of a diet, ileal digesta and faeces sample for 16, 24, 48 or 72h.

Amino acid	Correction factor
Lysine	1.09
Serine	1.04
Isoleucine	1.21

[*1] Correction factors determined by expressing the maximium amino acid yield as a proportion of the yield at 24h.

for lysine and isoleucine found in the present study were also similar to those reported by Kohler and Palter (1967), and for serine, threonine and isoleucine, the curves were similar to those reported by Tkachuk and Irvine (1969).

Correction factors reported for serine, isoleucine and threonine in foodstuffs of 1.08, 1.08 and 1.04 respectively, by Kohler and Palter (1967), of 1.05, 1.10, 1.08 and 1.07 for threonine, serine, valine and isoleucine respectively (Slump, 1980) and of 1.02-1.08 for threonine, 1.02-1.15 for isoleucine and 1.06-1.14 for serine, by Tkachuk and Irvine (1969) are similar to those found in the present study. These correction factors were determined in the same way as in the present study, except that serine and threonine values were extrapolated back to zero time to obtain the maximum values. These studies did not give correction factors for the other amino acids as they were not found to change with hydrolysis time.

Studies involving the determination of amino acid digestibility, generally do not include the analysis of diet and digesta or faecal samples over different hydrolysis intervals to determine the maximum values for each sample, or even hydrolysis of representative samples to determine correction factors. This study, however, has shown that for some amino acids and samples, correction factors are necessary to obtain accurate results. The corrected and uncorrected apparent ileal digestibility values for glycine for example, were 66.1 and 73.4% respectively. The differences between the corrected and uncorrected digestibility values for the other corrected amino acids, however, were less than 2 percentage units. Practically, therefore, and when calculating digestibility values, the use of correction factors may not be necessary. When accurate absolute values for the amino acid composition of a sample are required, however, correction should be undertaken.

Several workers (Kohler and Palter, 1967; Garnett, 1985) have suggested that the hydrolysis of each protein source should be

treated as a separate problem, and general correction factors cannot be used. Correction factors specific to each protein source need to be defined and applied. Multiple hydrolyses of numerous samples is very time consuming and costly, however, and the use of correction factors applied after one hydrolysis time (24h) is useful and at least makes some correction for losses or incomplete hydrolysis after 24h.

SECTION 2.2. ALKALINE HYDROLYSIS FOR THE DETERMINATION OF TRYPTOPHAN IN BIOLOGICAL SAMPLES.

a. INTRODUCTION.

There have been difficulties with the routine determination of tryptophan in proteins. Tryptophan is usually completely destroyed by the acid hydrolysis step in amino acid analysis, so various other methods of hydrolysis have been used which may give higher tryptophan recoveries. These include the addition of thioglycolic acid to HCl (Matsubara and Sasaki, 1969), or use of p-toluene sulphonic acid (Liu and Chang, 1971). These methods, however, cannot tolerate the high levels of carbohydrate found in some feedstuffs. Alkaline hydrolysis reportedly gives recoveries of tryptophan, proline, valine and leucine of 95-100%, although there are variable losses of all other amino acids with complete loss of aspartate, threonine, serine and cysteine (Davies and Thomas, 1973). Several different methods of alkaline hydrolysis have been reported, and there is much debate over the choice of alkali for hydrolysis. Sodium hydroxide (NaOH) has often been used for hydrolysis, and a variety of protecting agents have been used in addition to NaOH to enhance tryptophan recoveries. Brand and Kassell (1939) have shown that there is little advantage of using NaOH-sodium stannite over NaOH for hydrolysis of pure proteins, but Lucas and Sotelo (1980) have shown that NaOH-$SnCl_2$ gave higher tryptophan recoveries from lysozyme and skim-milk powder, and lithium hydroxide (LiOH) was better still. Nielsen and Hurrell (1985), however, concluded that there was no significant difference between tryptophan recovery from casein using NaOH or LiOH. Whitacre and Balscukat (1987) claimed to get tryptophan recoveries in excess of 94% using 4.3M LiOH. Barium hydroxide has also been used for hydrolysis (Miller, 1967) but this method requires precipitation of Ba^{2+} after hydrolysis, which may result in loss of tryptophan (Hugli and Moore, 1972). Lucas and Sotelo (1980) have shown that 2.5M and 4M barium hydroxide hydrolyses give much lower tryptophan determinations than hydrolysis

using NaOH or LiOH. Therefore it appears that either LiOH or NaOH is the best hydrolysing agent.

Starch has been used by several authors as a protecting agent in alkaline hydrolysis, leading to high recoveries of tryptophan from pure proteins (Hugli and Moore, 1972; Nielsen and Hurrell, 1985). High recoveries of tryptophan have also been obtained in the presence of glucose and fructose (Spies and Chambers, 1948; 1949). Lugg (1938) found that the presence of various carbohydrates when hydrolysing proteins in alkali did not reduce the tryptophan recovery. Therefore, good recoveries of tryptophan should be obtained from feedstuffs with high carbohydrate content, under alkaline hydrolysis.

Some workers have used internal standards to correct for the losses of trytophan occurring during alkaline hydrolysis, and several different substances have been investigated. The standard 5-methyltryptophan appears to give the best results (Nielsen and Hurrell, 1985). The use of internal standards does however assume that the standard behaves in the same way as the amino acid being determined. Nielsen and Hurrell (1985) found that corrections using 5-methyltryptophan tend to overestimate for tryptophan losses, as the recovery of 5-methyltryptophan was slightly lower than that of protein-bound tryptophan. Further, these workers found that 5-methyltryptophan is less stable upon storage than tryptophan, especially in acid conditions, which may lead to further overcorrection. Care needs to be taken, therefore,to ensure that hydrolysates are analysed as soon after hydrolysis as possible. Hugli and Moore (1972) have shown that upon storing hydrolysates at pH 2.2 (-20°C) there is a loss of 2.3% of tryptophan after 40h, and up to 9% at room temperature after 40h. They compared this with adjusting to pH4.5, and found that there was less than 1% loss of tryptophan after 40h at room temperature.

Normally, protein hydrolysis for tryptophan determination is conducted in glass hydrolysis tubes sealed under vacuum. However,

Nielsen and Hurrell (1985) have found that tryptophan is relatively insensitive to the presence of oxygen during alkaline hydrolysis. They found a decrease of only 10% in tryptophan when going from hydrolysis under high vacuum to no vacuum. This information may be useful when considering the use of screw-cap teflon containers instead of sealed tubes for hydrolysis of samples in alkali. The containers cannot be evacuated, but instead nitrogen gas is bubbled through the sample/alkali mixture, and onto the top of the mixture to expel any air from the container before fitting the lid. The main advantage from using this procedure is that a greater volume of alkali can be used for hydrolysis, and the increased alkali to sample ratio may improve the tryptophan recoveries.

In this study, preliminary work established that for a non-pure protein sample, such as a diet sample, a sample size of 50-100mg hydrolysed in 10ml of alkali in sealed evacuated tubes, gave the greatest yield of tryptophan. The difference between duplicates was below 5% of the mean value. It was also found that 4.3M LiOH gave up to 30% more tryptophan than 4.3M NaOH from a diet sample. Furthermore, the extent of hydrolysis was determined to be the same for LiOH and NaOH, according to the values obtained for proline, valine and leucine (which are 95-100% recovered after alkaline hydrolysis; Davies and Thomas, 1973). The values for these amino acids were the same for both alkalis, and were a little lower than the values obtained after acid hydrolysis of the same sample. LiOH was therefore chosen as the most suitable hydrolysing agent for use in the present study.

When sealed evacuated tubes were used, a 24h hydrolysis period gave maximum yields of tryptophan. Nielsen and Hurrell (1985), however, found that a 20h hydrolysis period at 110°C gave the highest tryptophan values for alkaline hydrolysis of casein. The hydrolysis time required to maximise tryptophan yield, therefore, may differ for different sources of material.

The aim of the present study, therefore, was to investigate the recovery of tryptophan from a pure protein lysozyme, which has a known tryptophan content, under certain hydrolysis conditions. A comparison was made between using the sealed tube method and teflon containers. The use of the containers allows a greater volume of alkali to be used which may improve the yield of tryptophan, and so was investigated using both the pure protein and a diet sample, over various hydrolysis times. The use of the internal standard 5-methyltryptophan was also investigated, and its recovery over different hydrolysis times compared with the recovery of tryptophan from lysozyme.

A second part of this study used the method found to be the most suitable for tryptophan determinations, and investigated the tryptophan yields obtained over different hydrolysis times, for diet, ileal digesta and faeces samples.

b. METHODS AND MATERIALS.

REAGENTS.

All reagents used were of AnalaR quality.
Lithium hydroxide (LiOH), Ajax Chemicals, Australia.
Sodium hydroxide (NaOH), BDH, England.
Lysozyme, Sigma Chemical Co., USA.
DL-Tryptophan, BDH, England.
5-methyl DL-tryptophan, Sigma Chemical Co., USA.

CHEMICAL ANALYSIS.

PART 1. COMPARISON OF SEALED TUBES WITH TEFLON CONTAINERS FOR 4.3M LiOH HYDROLYSIS OF LYSOZYME AND OF A DIET SAMPLE FOR THE DETERMINATION OF TRYPTOPHAN.

Lysozyme: Duplicate 10mg samples of lysozyme were accurately weighed into hydrolysis tubes (30ml) and teflon containers (30ml Tuf-tainer vials, Pierce, U.S.A.). One ml of 4.3M LiOH containing 10µmoles of 5-methyltryptophan was added to the tubes and mixed, and 7.5ml of 4.3M LiOH containing 10µmoles of 5-methyltryptophan were added to the containers, and mixed. The tubes were evacuated and sealed under vacuum, while nitrogen gas was slowly bubbled through the mixture in the teflon containers for 1-2 minutes, and onto the top of the liquid, and the lid fitted immediately. The tubes and containers were then placed in a forced air oven at 110°C for 16, 20 and 24h. The tubes were cooled before being cracked open, and the contents of both the tubes and containers were quantitatively transferred to volumetric flasks and made up to a volume of 10ml. The hydrolysates did not need to be filtered, so 7ml were adjusted to pH4.5 using concentrated hydrochloric acid, then made up to a volume of 10ml, and stored at -20°C. The amino acid analyser available for this study usually requires hydrolysates to be adjusted to pH3.5 prior to loading with the first buffer which has a pH of around 3.5. However,

tryptophan elutes with the third buffer on the analyser which has a pH of around 6.5, therefore if only tryptophan, and 5-methyltryptophan are required, only buffer three needs to be run on the analyser, so allowing the samples to be adjusted to pH4.5 prior to loading with the second buffer, and also resulting in the length of run on the analyser being considerably shortened.

After being adjusted to pH4.5, the sample (500µl) was loaded onto a Beckman 119 BL amino acid analyser, which had been calibrated with a standard mixture of amino acids, and a rapid screening analysis performed. Once adjusted to pH4.5, the analysis was carried out as soon as possible (usually within 48h) to avoid any tryptophan destruction. The positions of elution of tryptophan and 5-methyltryptophan are illustrated in Fig. 2.1.

Diet T: The diet sample was comprised of a mixture of meat, vegetables, fruit, dairy and cereal products. Duplicate 50-100mg samples of the homogenised, freeze-dried and finely ground diet were accurately weighed into large hydrolysis tubes and teflon containers. Ten ml of 4.3M LiOH containing 10µmoles of 5-methyltryptophan was added to the weighed diet sample in the hydrolysis tube and mixed, and 20ml of 4.3M LiOH containing 4µmoles of 5-methyltryptophan were added to the teflon containers. The tubes were sealed and containers purged with nitrogen gas as described above, and hydrolysis was carried out for 16, 20, 24 or 30h. The tubes were cooled before being cracked open, and the contents of the tubes and containers were quantitatively transferred to 25ml volumetric flasks and made up to volume. The hydrolysates were mixed then filtered through Whatman no.3 filter paper with the first 3-5ml discarded. Seven ml of the filtrate were adjusted to pH4.5, then made up to a volume of 10ml ready for loading onto the analyser, as described previously.

PART 2. INVESTIGATION OF THE HYDROLYSIS TIME REQUIRED FOR MAXIMUM TRYPTOPHAN VALUES FROM A DIET SAMPLE, AND ILEAL DIGESTA AND FAECES SAMPLES PERTAINING TO THAT DIET.

The diet, ileal digesta, and faeces samples were from those referred to in a subsequent section, and the details of the diet composition, and method of collection of the samples are given in Section 4.2. From these samples one each of diet, ileal digesta, and faeces were chosen, and used to determine the optimum time of hydrolysis for the analysis of tryptophan in such samples. Duplicate 50-100mg samples were accurately weighed into the teflon containers, and 20ml of 4.3M LiOH containing 4µmoles of 5-methyltryptophan were added. The containers were prepared for hydrolysis as described in part 1, and hydrolysis was carried out for 16, 18, 20, 24 or 30h. The hydrolysates were prepared for analysis and analysed as described in part 1 for the diet sample.

c. RESULTS.

PART 1. The position in which 5-methyltryptophan eluted overlapped slightly with the end of the ammonia peak, making accurate measurement of the peak difficult. The 5-methyltryptophan recoveries, and the corrected and uncorrected tryptophan values after 16, 20 or 24h for lysozyme, for the two methods are given in Table 2.5. The mean results for the tryptophan contents of Diet T after being corrected for by 5-methyltryptophan recovery, for the two methods, are given in Table 2.6.

The recovery of 5-methyltryptophan was higher with the teflon container method after hydrolysis of both the lysozyme and Diet T, as were the absolute tryptophan values for both samples. The agreement between duplicate analyses was similar after hydrolysis by the two methods, for both samples, and the difference between each pair of duplicates (expressed as a proportion of the mean) was generally less than 5% and always less than 10%.

The maximum tryptophan values were obtained after 20h hydrolysis for lysozyme, while a 24h hydrolysis time appears to give the greatest tryptophan results for Diet T. This highlights the need to investigate the hydrolysis time required to give maximum results when using different types of samples.

PART 2. The mean corrected results for tryptophan yield for each of the samples after 16, 18, 20, 24 or 30h hydrolysis are given in Table 2.7. The greatest tryptophan values were obtained after 20h hydrolysis for all three types of samples, and the agreement between duplicates was similar for all three materials with less than 5% difference in each case.

Table 2.5. The recovery of 5-methyltryptophan (%), uncorrected and corrected*1 tryptophan content (g/100g protein) in Lysozyme after 16, 20 and 24h hydrolysis in 4.3M LiOH in sealed hydrolysis tubes and teflon containers.

	Hydrolysis time (h)		
	16	20	24
Sealed tubes			
5-methyltryptophan (%)*2	93.9	91.9	90.0
Tryptophan (g/100g)			
uncorrected	6.6	6.8	6.1
corrected	7.0	7.4	6.8
Teflon containers			
5-methyltryptophan (%)*2	97.3	96.4	93.8
Tryptophan (g/100g)			
uncorrected	7.1	8.0	7.5
corrected	7.3	8.3	8.0

*1 Corrected for the recovery of 5-methyltryptophan.

*2 Recovery of 5-methyltryptophan.

Table 2.6. 5-methyltryptophan recoveries (%) after 16, 20, 24 and 30h hydrolyses of a diet sample in 4.3M LiOH, and the uncorrected and corrected tryptophan values (nmol/mg freeze-dried sample) at these times using sealed hydrolysis tubes and teflon containers.

	Hydrolysis time (h)			
	16	20	24	30
Sealed tubes				
5-methyltryptophan (%) *1	92.8	90.8	91.0	88.0
Tryptophan (nmol/mg)				
uncorrected	10.8	10.9	12.3	8.2
corrected*2	11.6	12.0	13.5	9.3
Teflon containers				
5-methyltryptophan (%) *1	93.0	89.9	90.3	89.0
Tryptophan (nmol/mg)				
uncorrected	13.2	13.1	14.3	12.3
corrected*2	14.2	14.6	15.8	13.8

*1 Recovery of 5-methyltryptophan.

*2 Corrected for the recovery of 5-methyltryptophan.

Table 2.7. Corrected[*1] tryptophan determination (mg/100g freeze-dried sample) of diet, ileal digesta and faeces samples after 4.3M LiOH hydrolysis in teflon containers after 16, 18, 20, 24 or 30h.

	Hydrolysis time (h)				
	16	18	20	24	30
Diet sample	138.5	152.8	173.2	108.6	93.3
Ileal digesta	177.7	184.4	217.9	172.4	129.3
Faeces sample	282.9	299.8	325.1	275.3	275.7

[*1] Corrected for the recovery of 5-methyltryptophan.

d. DISCUSSION.

When analysing samples that consist of non-homogeneous materials, it is important to have good agreement (< 5% difference of mean value) between duplicate samples, and to have reproducibility of results. In this study both of these requirements were met with the teflon container method.

The use of teflon containers for alkaline hydrolysis of both a pure protein and feedstuffs gave greater tryptophan yields at each hydrolysis time than when the same samples were hydrolysed under high vacuum in sealed tubes. It appears, therefore,that the effect of not evacuating the hydrolysis vessel has been overcome by the increased alkali to sample ratio. The results indicate that there was either a decreased destruction of tryptophan in protein and/or an increased hydrolysis of protein with the higher alkali to sample ratio. The teflon container method was also simpler, faster, and with the containers being reusable, the method would also be less expensive for routine analysis.

The literature value for the tryptophan content in lysozyme from protein sequencing is 8.56g/100g of protein (Daynoff, 1973), therefore the recovery of tryptophan from lysozyme was low for the sealed tube method, only 86%, even after correction. The recovery of 93.5% of the literature value for tryptophan in lysozyme using the teflon container method was higher than the uncorrected tryptophan value reported by Nielsen and Hurrell (1985) of 89.0%, although the corrected values were similar, 97.0% and 98.4% respectively. H. Voon (pers. comm.) recovered 81.5% of tryptophan using a method similar to that used in the present study, but Lucas and Sotelo (1980) only recovered 69% of tryptophan from lysozyme after 20h hydrolysis at $100^{o}C$ in 4M LiOH. From the results of the present tryptophan recovery experiments it is assumed, therefore, that the tryptophan values obtained from analysis of samples with unknown tryptophan content are 90-100% of the true value.

The use of the internal standard 5-methyltryptophan was also examined and the recovery was higher for the teflon container method. Its recovery was generally greater than 90% (which was similar to the greatest uncorrected recovery obtained for tryptophan in lysozyme, 93.5%). This meant that the use of the internal standard allowed only a small adjustment to the tryptophan values. The recovery curves are similar to those reported by Nielsen and Hurrell (1985), and the calculated tryptophan values using these recoveries also resemble their results. The 5-methyltryptophan was progressively destroyed with the increasing hydrolysis time, and so the correction becomes more important as the hydrolysis interval is increased. Miller (1967) investigated the recovery of added L-tryptophan over several hydrolysis times with $Ba(OH)_2$, and also found that the recovery decreased over time, so used this to correct the tryptophan values obtained for foodstuffs. It would, however, be more desirable to use an internal standard.

Nielsen and Hurrell (1985) reported that 5-methyltryptophan recovery was generally lower than that of protein-bound tryptophan, but the reverse was found in the present study, with 5-methyltryptophan being destroyed relatively more slowly than tryptophan. Therefore the tryptophan values calculated in the present study may be underestimated, even when corrected using the recovery of 5-methyltryptophan. However, this correction would be better than none. It should also be noted that the corrected tryptophan values decrease with hydrolysis time after reaching the maximum value, which suggests that the internal standard is not allowing for complete correction of the losses during hydrolysis. This also emphasises the need to investigate the hydrolysis time required to give the greatest tryptophan yields with unknown samples even when using internal standards.

The recovery of tryptophan from lysozyme when using the teflon container was greatest after 20h, although for Diet T the greatest

values were obtained after 24h. The hydrolysis times required to give maximum tryptophan values were determined for the types of samples described in a subsequent section of this thesis. It was found that a 20h hydrolysis time gave the maximum yields of tryptophan from diet, ileal digesta and faecal samples. The analysis of feedstuffs and digesta samples for tryptophan requires the analyst determine the conditions of hydrolysis and processing that give maximum results for the particular samples being analysed. This study has shown how different types of samples, whether pure protein, digesta samples or two different diet combinations, behave differently under the same hydrolysis conditions, and may require different times of hydrolysis to give the greatest tryptophan values.

For the other studies reported in this thesis, tryptophan was determined after hydrolysis of 50-100mg samples of diet and digesta in 4.3M LiOH for 20h at 110°C using teflon containers, and losses corrected for by the recovery of the internal standard 5-methyltryptophan.

2.3. OVERALL CONCLUSION.

From the work reported in this chapter it is concluded that for the accurate determination of all amino acids in diet, ileal digesta and faeces samples, different conditions of hydrolysis are required for the different amino acids. The determination of all common amino acids except methionine, cysteine and tryptophan may be successfully carried out after acid hydrolysis in sealed evacuated tubes. Correction may need to be made for incomplete hydrolysis or destruction that may occur, for the individual amino acids, after a single 24h hydrolysis. Methionine and cysteine should be determined as methionine sulphone and cysteic acid respectively after performic acid oxidation of the sample followed by acid hydrolysis under reflux conditions using round-bottomed flasks. Finally, tryptophan determination may be performed following alkaline hydrolysis in teflon containers for 20 hours. Correction for losses may need to be made by including the internal standard 5-methyltryptophan.

CHAPTER 3.

CELITE AS A NON-DIGESTIBLE MARKER IN DIGESTIBILITY STUDIES WITH HUMANS.

3.1. INTRODUCTION.

Digestibility studies which do not involve total collection of faeces or ileal digesta, but rather sampling of the material, rely on the use of a non-digestible marker. The latter may be used to relate the concentration of a component in the samples collected to that in the diet.

Traditionally, chromic oxide has been used in human studies (Branch and Cummings, 1978; Hesp et al., 1979; Bacon, 1980). Acceptance of such a compound by the subjects involved in the trial, however, may sometimes be a problem. Chromic oxide does not occur naturally in the diet of humans, and it is bright green so colouring the diet when added as a marker. A natural marker occurring in the diet, such as acid-insoluble ash (Shrivastava and Talapatra, 1962; McCarthy et al., 1974; Yen et al., 1983), may be more acceptable than an added compound. Natural acid-insoluble ash has been used in digestibility studies with pigs (Wunsche et al., 1984; Sugimoto, 1984) and high recoveries of 90-100% have been reported. Before the conduct of the present study, however, an investigation (Rowan, unpublished) of the acid-insoluble ash levels in several common food ingredients for humans showed that the levels are too low for its use as a marker. Also, the recovery of natural acid-insoluble ash in the faeces of simple stomached animals may not always be complete (Schrama pers. commun.). The latter two factors have led to the investigation of the suitability of using added acid-insoluble ash (Celite, a diatomaceous earth) as a marker compound. Celite is a bland, non-toxic, non-digestible compound which has the advantage of being white rather than coloured. Celite added to the diets of pigs (McCarthy et al., 1974; 1977) and chickens (Vogtmann et al., 1975)

has been used as a marker in digestibility studies, with high recoveries of the acid-insoluble ash being reported.

The aim of the presently reported work was to investigate the suitability of added acid-insoluble ash as an indigestible marker for studies with humans where near-complete but not total collection of excreta is obtained. Three trials are reported. In the first experiment the faecal recovery of Celite added to a human-type diet fed to pigs, was determined. The collection of faeces was carried out for 3 days, because a 3-day collection period is often regarded as the minimum length of time required to give reliable results with human subjects. The study also examined extending the collection period by 3 days to determine if a 3-day period would indeed be adequate. The pig was used, to allow the faecal recovery of Celite to be determined, without subjecting humans to experimentation unnecessarily. The pig was chosen as a model animal in this study because it allows quantitative collection of faeces to be made and dietary intake can be controlled. The diet used comprised ingredients commonly eaten by humans.

The second trial involved determining the faecal recovery of added Celite in human subjects over a 5-day faeces collection period. This was an acceptable collection period for the subjects involved in the trial.

The third trial investigated the recovery of added Celite at the terminal ileum of ileostomised pigs, fed a single meal of starch-based biscuits. The ileal output was collected for 24h. It was assumed that after 24h all of the meal eaten would have reached the terminal ileum.

3.2. ANIMALS, DIETS AND PROCEDURE.

a. Trial 1. Five six-week-old 12kg liveweight Large White x (Landrace x Large White) entire-male pigs were housed individually in metabolism crates and kept at a constant temperature of 24$\pm$ 1°C.

A diet was formulated which consisted largely of ingredients consumed by humans (Table 3.1), and Celite was added as a non-digestible marker. The pigs were weighed at the beginning of the trial, and fed on a dry-matter basis at a set level of 0.08 of body weight ($kg^{0.75}$).

After a 2-day preliminary period in the new surroundings, the animals were fed the diet twice daily (0830h and 1630h) for seven days. Total collection of faeces was made daily over the last 3 days with the faeces being frozen (-20°C) immediately. All refusals and spillages of the diet were collected. Representative samples of the diet were taken and frozen (-20°C) for subsequent chemical analysis. The diet was fed to the same pigs for a further 3 days with separate daily collection of total faeces over these days, for investigation of acid-insoluble ash recovery over a longer period.

b. Trial 2. Six adults (3 women and 3 men) between the ages of 22 and 60 followed a set diet for seven days, with total faeces collection over the last 4 days of diet consumption and including the following day, the eighth day. The composition of the diet is shown in Table 4.1 in Section 4.2 of this thesis. Each subject chose one diet out of diets A, B or C (Table 4.1). Diets A and B included one gelatin capsule (Parke, Davis and Co., Australia) containing 0.58g and 0.87g of Celite respectively, to be taken with the morning and evening meals. Diet C included three gelatin capsules per day containing 0.77g each; one to be taken with the morning, midday, and evening meals. Celite was consumed at a constant level of daily intake (0.35% of dry-matter eaten for subjects 1 to 4, 0.47% for subject 5, and 0.23% for subject 6). The different intakes across

Table 3.1. Ingredient composition of a diet fed to young pigs to determine the recovery of Celite as acid-insoluble ash.

Ingredient	Composition (g/kg dry-matter)
Meal mixture[*1]	227.0
Breadcrumbs	364.0
Ground wheat	297.0
Skim milk powder	85.0
Maize oil	20.0
Vitamin/mineral premix[*2]	1.0
Celite	6.0

[*1] An homogenised mixture of meat, vegetables, fruit, dairy products and cereals.

[*2] Tasmix, pig grower Vitamin/Mineral premix (Tasman Vaccines Ltd., Auckland, N.Z.).

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subjects was because the latter two subjects decided to change their dietary level of consumption after day 1, but kept the original level of Celite intake.

Total collection of faeces was made from 0800h on day 4 to 0800h on day 9, with the faeces being frozen (-20°C) within half an hour of collection.

c. Trial 3. Six 25-30kg liveweight Large White x (Landrace x Large White) entire-male pigs which had previously been surgically prepared with ileostomies (refer Section 4.2), were housed individually in metabolism crates and kept at a constant temperature of 22$\pm$1°C.

The pigs were fasted overnight for 15 hours, then the ileostomy bags changed. The animals were given a meal of protein-free biscuits (Section 5.2., Table 5.1), which contained 5g/kg dry-matter of Celite as a non-digestible marker. Each pig received 200$\pm$ 1g of the biscuits, and water was provided *ad libitum*.

Digesta were collected for 24h with the contents of the ileostomy bags being emptied every 3h during the day but with the last 8h collection being emptied the next morning. The collection for the first 6h was kept separate from that for the last 18h. During collection, the samples were refrigerated until the 6 and 18h periods were finished, at which time they were weighed and frozen (-20°C).

3.3. CHEMICAL AND STATISTICAL ANALYSIS.

The faeces and diet samples were removed from the freezer, thawed, weighed, mixed, and representative sub-samples were taken for dry-matter determinations on the wet material. The remainder was freeze-dried and ground (1mm) for further analysis.

The wet and freeze-dried diet and faecal samples were analysed for dry-matter by drying to a constant weight in a forced air oven at 95°C, and for acid-insoluble ash levels using a method based on that of McCarthy *et al.* (1974). Seven to 10g of ground freeze-dried material were boiled in 100ml of 4M HCl for 1h, then filtered through ashless filter paper and washed with boiling water. In trial 3 only 1-3g of freeze-dried material was used because very little material was collected in the 6h period. The samples were then oven-dried (100°C) overnight, and ashed at 500°C for at least 24h.

The digestibility of dietary dry-matter (DM) was calculated either on the basis of total collection of faeces or on the acid-insoluble ash (AIA) concentration in the diet and faeces, using the following respective equations:

$$\text{Digestibility of dry-matter (\%)} = \frac{\text{total } DM_D^{*1}\text{ (g)} - \text{total } DM_F^{*2}\text{ (g)}}{\text{total } DM_D^{*1}\text{ (g)}} \times \frac{100}{1}$$

$$\text{Digestibility of dry-matter (\%)} = \frac{\dfrac{DM_D\text{ (g/100g)}}{AIA_D\text{ (g/100g)}} - \dfrac{DM_F\text{ (g/100g)}}{AIA_F\text{ (g/100g)}}}{\dfrac{DM_D\text{ (g/100g)}}{AIA_D\text{ (g/100g)}}} \times \frac{100}{1}$$

*1 DM_D or AIA_D = dry-matter or acid-insoluble ash in diet in both equations.

*2 DM_F or AIA_F = dry-matter or acid-insoluble ash in faeces in both equations.

For Trial 1, a statistical model including terms for method and time was fitted to the dry-matter digestibility determinations, and the data were subjected to an analysis of variance (Snedecor and Cochran, 1982). The term for method in the model referred to digestibility calculated by total collection of faeces or by reference to acid-insoluble ash. The term for time referred to the first 3-day versus second 3-day period (analysis 1), or 3 versus 6-day periods (analysis 2).

The effects of three versus six days, and the first 3-day versus the second 3-day collection periods were examined for Trial 1. In Trial 2, the means of dry-matter digestibility calculated by the two methods were compared using a paired t-test (Snedecor and Cochran, 1982).

3.4. RESULTS AND DISCUSSION.

The recoveries ($\bar{X}\pm$S.E.) of acid-insoluble ash for the first 3-day and total 6-day faeces collections in the pig, the 5-day faeces collection in humans, and the ileal collection in the pig are given in Table 3.2. The dietary dry-matter digestibility coefficients determined by either total faeces collection or by reference to acid-insoluble ash are given in Table 3.3.

The percentage recovery of added Celite found in the present study was lower than 100%. However, as shown in Appendix 2, Table 2.1, marker recoveries ranging from 80% to 100% have only a minor effect on the estimation of apparent digestibility.

The mean apparent digestibilities of dry-matter for the total collection or marker methods were not significantly different ($P<0.05$). For details of the full statistical analysis see Appendix 2, Table 2.2. The standard errors for the recoveries and digestibilities were low and similar for all three trials, and were also similar for dry-matter digestibility calculated by the total collection or marker methods.

The acid-insoluble ash recovery for one of the animals (pig 5) in Trial 1 was greater than 100%, and this was confirmed by repeating the chemical analyses. If this recovery value was excluded from the data set, there was a significant difference ($P<0.01$; refer Appendix 2, Table 2.3) between the two mean estimates of apparent dry-matter digestibility, but the difference remained very small (1.24%) in absolute terms.

The finding of similar digestibility estimates determined using either total faeces collection or by reference to the added marker Celite is in agreement with other workers using pigs (McCarthy _et al._, 1974; 1977) and chickens (Vogtmann _et al._, 1975).

Table 3.2. The recovery of added Celite ($\bar{X}$±S.E.) in faeces of young pigs (n=5; 12kg) and adult humans (n=6) or ileal output of pigs (n=6; 25kg) consuming diets consisting of ingredients commonly eaten by humans.

Trial			Recovery
1*1	1st	3d	90.6 (±2.24)
	2nd	3d	89.4 (±6.42) *2
	Total	6d	90.0 (±2.24) *3
2*4			92.1 (±4.59)
3*5			93.5 (±5.70)

*1 Collection of faeces from 12kg pigs for the 1st and 2nd 3-day periods and the total 6 days collection respectively.

*2 There was no significant difference between the 1st 3-day and 2nd 3-day collection periods of faeces for the recovery of acid-insoluble ash.

*3 There was no significant difference between the 1st 3-day and total 6-day collection of faeces for recovery of acid-insoluble ash.

*4 Collection of faeces from adult humans for a 5-day period.

*5 25-30kg pigs; collection of ileal output.

Table 3.3. The apparent dry-matter digestibility (X±S.E.) for young pigs (n=5; 12kg) or adult humans (n=6) given a human-type diet, after total collection of faeces or by reference to a non-digestible marker.

Trial		Apparent digestibility of dry-matter		Level of significance
		Acid-insoluble ash[*1]	Total collection	
1	1st 3d	92.4±0.28[*2]	93.3±0.25	NS
	2nd 3d	91.7±0.48[*3]	92.3±1.05	NS
	Total 6d	92.1±0.37	92.8±0.58	NS
2		92.7±0.69	93.3±0.55	NS

[*1] Apparent digestibility based on the acid-insoluble ash content of an aliquot of the total faeces.

[*2] There was no significant difference between the 1st 3-day and total 6-day collection periods for the calculation of dry-matter digestibility using either method.

[*3] There was no significant difference between the first 3-day and second 3-day collection periods for the calculation of dry-matter digestibility using either method.

In digestibility studies using human subjects, problems are often encountered with extended periods of collection. When the collection period in Trial 1 was extended to 6 days (i.e. the results for the first and second 3-day periods combined), neither the recovery of acid-insoluble ash, nor the dry-matter digestibility estimates altered significantly. Also there was no significant difference ($P<0.05$) between estimates of digestibility for each method between 3- and 6-day collections. It was concluded, therefore, that collection of faeces for longer than three days does not lead to an increased recovery of added acid-insoluble ash or an improvement in the estimation of dry-matter digestibility. Based on this evidence, a faeces collection period of at least three days would be acceptable for application to studies involving human subjects.

Another problem related to digestibility studies is chosing a period of time for dietary adjustment that will allow collection of representative faeces samples, while also being acceptable to the subjects involved. A finding of the present study was that there was no significant difference between acid-insoluble ash recoveries or apparent dry-matter digestibility coefficients determined by the two methods, when the first 3-day and second 3-day periods were analysed separately. This suggests that the 4-day dietary adjustment period was sufficient to ensure that representative samples were collected, and that there would be no advantage of extending the adjustment period.

The results of the present study show that Celite (acid-insoluble ash) can be used as a marker in digestibility studies with young pigs given a diet similar in composition to that consumed by humans and where near-complete collection of excreta is obtained. Celite has not been used before as a marker in digestibility studies involving human subjects. It was concluded that Celite (acid-insoluble ash) recovery in human subjects is satisfactory, and it can be used in future work as a marker to estimate digestibility coefficients in trials involving humans. A further conclusion is

that Celite recovery in ileostomised pigs is satisfactory. Based on the latter result and the high recovery of acid-insoluble ash in human faeces it is assumed that Celite may be used as a marker when collecting samples of ileal digesta from humans, wherever near-complete collection of excreta is obtained. The present study did not examine the variation in acid-insoluble ash levels in spot samples of excreta.

CHAPTER 4.

THE DETERMINATION OF APPARENT ILEAL AND FAECAL AMINO ACID DIGESTIBILITY VALUES IN ADULT HUMANS RECEIVING A MIXED DIET, AND COMPARISON WITH VALUES DETERMINED WITH THE GROWING PIG.

4.1. INTRODUCTION.

The determination of protein and amino acid digestibility in domestic farm animals is now often carried out using the ileal assay, which is preferred over the traditional faecal method (Low, 1980a; Rerat, 1981). However, no studies with humans have yet been undertaken to formally establish that the ileal method for determining amino acid digestibilty is more accurate than the faecal method. Results from studies involving other monogastric animals (Payne *et al.*, 1968; Varnish and Carpenter, 1975; Low, 1982a) suggest that a difference between the ileal and faecal values probably also exists for humans. Furthermore, studies involving human subjects (Sammons, 1961; Gibson *et al.*, 1976; Schrimshaw *et al.*, 1983; Istfan *et al.*, 1983; Wayler *et al.*, 1983; Young *et al.*, 1984; Sandstrom *et al.*, 1986) in which nitrogen (N) in the faeces or ileal output has been determined, indicate that the two methods do lead to different results. The present study which involved the total collection of faeces and ileal digesta aimed to compare ileal and faecal estimates of amino acid digestibility for humans receiving a balanced mixed diet.

A second aim of this study was to evaluate the growing pig as a model for determining dietary amino acid digestibility in humans. The digestibility of dry-matter at the ileum and in faeces, and of gross energy in the faeces, were also determined for both species, and the respective values compared between species.

4.2. METHODS AND MATERIALS.

a. Human study.

Ethics approval was obtained from the Auckland Hospital Human Ethics Committee and the Massey University Human Ethics Committee, before the onset of the trial and all subjects gave their consent with full knowledge of the experimental procedure. Six healthy adult Caucasian volunteers (two women, four men; details of each subject are given in Appendix 3, Table 3.1) with well-established ileostomies (> one year), after total surgical removal of the large intestine and minimal resection of the small intestine (Brooke, 1952), were given a fixed diet (Table 4.1) for seven days. These subjects had undergone ileostomy operations due to ulcerative colitis. One person was interviewed before commencement of the trial, to allow design of a diet that was acceptable to an ileostomate. The other five subjects also agreed to eat the foods nominated by subject one. Three different dietary consumption levels were offered to the subjects and they chose the one that they considered to be closest to their normal level of consumption. The gross daily nutrient intakes of each subject determined by reference to food-table values are given in Table 4.2.

The food was prepared before the seven day experimental period, with individual meal portions of each ingredient being weighed and packaged separately. The vegetables were weighed ($\pm$1.0g of required weight) and supplied raw or frozen. These were subsequently boiled or steamed by each subject, and care was taken not to overcook any food. The chicken was skinless white breast meat and each subject was allowed 5g of oil to cook this in. The apples, tomatoes and bananas were weighed and only those within $\pm$10g of the required weight were used, with the exact weight being recorded. These were eaten in the fresh state. Each subject recorded the amounts of sugar and lemonade consumed each day at the end of the seven day period (refer Appendix 3, Table 3.2), and this information was used to

Table 4.1. Three alternative diets followed by 12 human subjects, and that (diet C) given to 12 pigs.

Meal	Food	Diet A	Diet B	Diet C
		Amount consumed (g)		
Breakfast (0700h)	Toast (wholemeal)*1	60	90	120
	Butter	10	15	20
	Marmalade *2	10	15	20
Lunch (1200h)	Bread (wholemeal)*1	49	74	98
	Butter	10	15	20
	Tomato *3	110	160	220
	Sao crackers *4	10	15	20
	Cheddar cheese	45	68	90
	Banana *5	150	225	300
Afternoon tea	Plain biscuit *6	8	12	16
Dinner (0600h)	Chicken	100	150	200
	Potato (peeled)	100	150	200
	Carrots (peeled)	100	150	200
	Frozen peas *7	60	90	120
	Frozen beans *7	70	105	140
	Apple *8	130	190	260
Daily allowance homogenised milk		300ml	450ml	600ml

Unlimited tea, coffee, lemonade, water and sugar*9.

*[1] Shand's bakery, Main Rd, Huntly, New Zealand.

*[2] Craig's Breakfast Marmalade. Butland Industries Ltd., 644 Great South Rd, Ellerslie, Auckland, N.Z.

*[3] Weighed and eaten with skin.

*[4] Arnott's Sao crackers. Arnott's Biscuits (N.Z.) Ltd, Saleyards Rd, Private Bag, Otahuhu, Auckland, N.Z.

*[5] Weighed with skin.

[6] Arnott's Superwine biscuits. Address as above ([4]).

*[7] Wattie's frozen peas and frozen beans. Wattie Frozen Foods Ltd, 89 Carbine Rd, Mt Wellington, Auckland, N.Z.

*[8] Red delicious apple weighed and eaten with skin.

*[9] In the studies involving faeces collection, sugar and lemonade were restricted; see Appendix 3, Table 3.4.

Table 4.2. Gross daily intakes of selected nutrients for the human subjects on 3 alternative diets.

Nutrient	Diet		
	A	B	C
	Daily intake*1		
Protein (Nx6.25;g)	65.7	98.6	131.4
Energy (kJ)*2	5600 (6000)	8400 (8900)	11200 (12000)
Dietary Fibre (g)	30.1	45.2	60.2
Fat (g)	52.2	78.3	104.4
Calcium (mg)	903.9	1355.9	1807.8
Iron (mg)	6.9	10.4	13.8
Vitamin C (mg)	64.2	96.3	128.4

*1 Calculated from Paul and Southgate (1978) and adjusted according to Wiles *et al.* (1980).

*2 Gross energy determined by bomb calorimetry; values in brackets for the diet used during the faeces collection.

estimate each subject's energy intake during the period. The five subjects who had chosen a diet kept a brief record of their activities and working hours, and with this information along with knowledge of their body weights and occupations, each subject's approximate energy expenditure during the trial was determined (refer Appendix 3, Tables 3.1 and 3.3). This was used to check retrospectively, if the diet chosen supported an energy intake commensurate with each subject's energy expenditure (Table 4.3). The diet ingredients were generally part of the normal diet of these subjects. After three days on the diet, ileostomy output was collected at regular intervals (approximately every 2-3h), over 24h periods from 0800h on the 4th day to 0800h on the 8th day. The ileostomy bag contents were emptied into a container with 10ml of 4M hydrochloric acid (HCl) added, and kept on ice for the 24h period. The material was then weighed and frozen.

A further six healthy adult Caucasian volunteers (three women, three men) were given a fixed diet of three meals/day for seven days (Table 4.1), but with restrictions on the allowances of sugar and lemonade. The food ingredients and their preparation were as described above, except that instead of oil allowed for cooking the chicken for the evening meal, each subject had to use 10, 15 or 20g of butter for diets A, B or C respectively. The subjects chose the level of consumption that they considered was closest to their normal level of intake. The daily energy intake was kept constant by restricting the amounts of sugar and lemonade allowed (see Appendix 3, Table 3.4), and the butter allowed for cooking had to be eaten. The approximate energy expenditures of these subjects were also estimated from information on their occupations, body weights, and levels of activity (Table 4.3). After three days on the diet, faeces were collected for the following five days from 0800h on the 4th day to 0800h on the 9th day. The samples were frozen as soon as possible, in four cases immediately, and for the other two usually within one hour of collection, and were weighed.

Table 4.3. Daily gross energy intakes of six ileostomates and six intact human subjects each on a constant diet for 7 days compared with their approximate daily energy expenditures.

Subject[*1]	Diet	Energy intake[*2] (kJ)[*4]	Daily energy output[*3] (kJ)
1	A	5700	-[*5]
2	A	6000	7800
3	B	8630	9930
4	B	10470	9380
5	C	12340	12300
6	B	9000	8820
7	B	8900	9600
8	B	8900	10180
9	C	12000	16000
10	A	6000	8500
11	B	8900	10500
12	B	8900	9200

[*1] Subjects 1-6 were ileostomates, 7-12 intact human subjects.

[*2] Calculated using tabulated values (Paul and Southgate, 1978).

[*3] Calculated using tabulated values (Durnin and Passmore, 1967), refer Appendix 3, Table 3.3.

[*4] These values take into account the sugar and lemonade consumed by each subject (refer Appendix 3, Table 3.2).

[*5] Energy expenditure not determined because diet was designed specifically to meet the requirements of this subject.

b. Animal study.

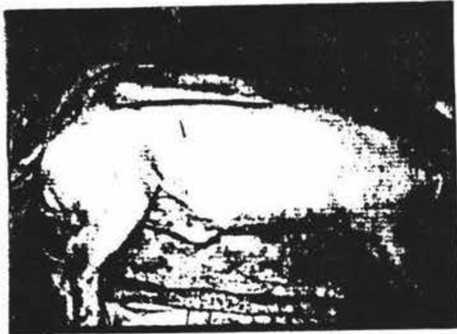
Six healthy 25kg liveweight entire male pigs (Landrace x (Landrace x Large white)) were surgically prepared with ileostomies (refer Fig. 4.1) using a method similar to that described by Brooke (1969). The pigs were given a pre-operative sedative (1-2ml of Stresnil; Smith Kline and French laboratories Ltd., Australia) intra-muscularly and then anaesthetised with halothane/oxygen (Fluothane, Imperial Chemical Industries Ltd., England) administered by mask. An oblique right lateral abdominal incision was made through which the stoma was to be formed. A 5cm incision was made through the skin, muscle layers and peritoneum using cutting quartery, 3cm posterior to the rib cage.

The ileocaecal junction was located and a loop of ileum was exteriorised as close to the junction as possible. Where the ileocaecal fold ended (5-10cm from the ileocaecal junction), the ileum and the common mesentery were divided between vascular arcades so that the ileal blood supply was preserved. The distal end was closed by suturing the serosal edges together and was then allowed to drop back into the abdomen.

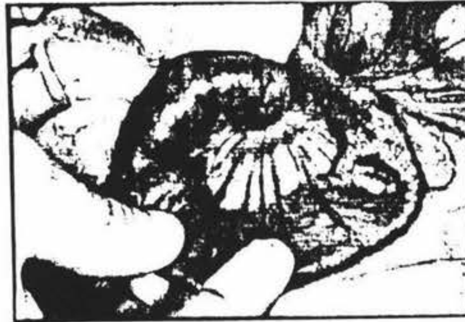
The cut edge of the mesentery was attached to the parietal peritoneum of the ventral abdominal wall to avoid internal herniation, leaving approximately 5cm of proximal ileum exposed for the formation of the stoma. A purse string suture was placed through the mesentery and parietal peritoneum of the right iliac fossa and of the ventral abdominal wall lateral to the incision.

The abdominal wall was closed in layers around the end of the ileum and relieving incisions were made if necessary in individual layers at right angles to the direction of the wound to avoid pinching of the stoma. A Duval lung-holding forceps was inserted into the ileal lumen and the intestinal wall grasped to form a fixed point so that the terminal portion could be everted. Finally the skin was sutured,

Figure 4.1. The ileostomy operation performed on the 25kg liveweight pig. (surgeon: Dr David Carr, Massey University, New Zealand.).



a. Ileostomy site.



b. Ileocaecal junction.

c. Division of the mesentery.

d. Transection of the ileum.

and at the stoma, the cut edge of mucosa was sewn directly to the skin with one suture being placed through skin, mucosa and mesentery, to fix the position of the everted portion to the underlying ileum. The pigs were disconnected from the halothane, and given an intra-muscular injection of antibiotics (5ml of Streptopen; Glaxo N.Z. Ltd., Palmerston North, N.Z.).

After the operations the pigs were kept singly in smooth-walled metabolism crates and were housed at a temperature of 22 $\pm$ 1°C, and given an homogenised diet consisting of a mixture of ingredients used in the experimental period of the present study, with solid food being introduced after two days. Care was taken to ensure that the pigs received adequate water to avoid dehydration, and salt to avoid Na^{+} depletion due to the ileostomy, both before and during the experimental period. Careful attention was also paid to cleanliness around the stoma and in the pig's environment.

After two weeks, the pigs appeared to have fully recovered from surgery. The six pigs were given a fixed diet of three meals/day for seven days as described for the human study except that all pigs received diet C, as this was closest to the diet they would receive if fed dry-matter at 10% of metabolic body weight ($kg^{0.75}$). The latter intake was considered suitable to support normal growth in the pig. The food was cooked for all pigs at each meal-time, and care was taken to ensure that the food was prepared in the same way as for the study involving human subjects. After three days on the diet, the ileostomy output was collected every 2-3h for 24h, from 0800h on the 4th day to 0800h on the 8th day, placed in a container containing 10ml of 4M HCl,and immediately chilled. At the end of each 24h period the contents of the containers were weighed and frozen.

Ileal digesta were collected from the pigs using an adapted human ileostomy collection apparatus (Combihesive, E.R. Squibb and Sons Ltd., N.Z.). A base plate was made using 2mm thick reinforced

rubber. The 38mm plastic flange was taken from the normal Karaya base plate and stitched and glued onto the rubber plate using thin nylon and Bostik glue (Bostik 2402, Bostik New Zealand Ltd.). The rubber plate was stitched to the pig's skin while the animal was under anaesthetic, after being glued in place with Sicomet glue (Henkel, Germany). A large piece of elastic band (15 cm wide) with a hole cut to fit over the flange, was wrapped around the girth of the pig to hold the base plate firmly to the side of the pig's body. This was to prevent leakage around the stoma, and to prevent the pig from catching the apparatus on the side of the metabolism crate. The collection bags were attached at the flange and changed every 2-3 hours.

A further six healthy 25kg liveweight entire male pigs (Landrace x (Landrace x Large white)) were kept singly in smooth-walled metabolism crates and were housed at a temperature of 22 $\pm$ 1°C. After an initial adjustment period, the pigs were given a fixed diet of three meals/day for seven days as described in the faeces collection part of the human study, with each pig receiving diet C. All the food was cooked at the same time for each day, and prepared in the same way as for the study involving the human subjects. After three days on the diet, faeces were collected for the following five days from 0800h on the 4th day to 0800h on the 9th day. The samples were weighed and frozen, immediately after collection.

CHEMICAL ANALYSIS.

Daily ileal digesta and faeces samples were each freeze-dried and pooled for each subject or animal, then finely ground and frozen for subsequent analysis.

During the study three samples of the daily diet were taken. These were weighed, homogenised, freeze-dried, finely ground (1mm), mixed and frozen for subsequent analysis. All reagents were of AnalaR quality, and were the same as those used in Chapter 2 of this thesis.

All samples were analysed for amino acids. Three hydrolysis procedures were used:

1. For all amino acids except methionine, cysteine and tryptophan: 6M HCl hydrolysis in sealed evacuated tubes for 24h at 110°C. Duplicate 50mg samples of the freeze-dried and finely ground diet, ileal digesta and faeces samples were accurately weighed into large (30ml) glass hydrolysis tubes, and 10ml of 6M HCl were added, and mixed. The tubes were evacuated and sealed, and placed in a forced air oven at 110°C for 24h. The hydrolysates were made up to 50ml in volumetric flasks, filtered through Whatman no.3 filter paper with the first 3-5ml discarded, and 2ml evaporated to dryness, and stored at -20°C for subsequent analysis.

2. For methionine sulphone and cysteic acid: 6M HCl hydrolysis of a performic acid-oxidised sample in round-bottomed flasks for 24h under reflux. Duplicate 50mg samples of the freeze-dried and finely ground diet, ileal digesta and faeces samples were accurately weighed into 250ml round-bottomed flasks. Two ml of the oxidation mixture (1.0ml of H_2O_2, 9.0ml of 88% formic acid and 50mg of phenol) were added to the sample in the flask and oxidation proceeded at 0°C for 16h, after which the excess performic acid was degraded by adding approximately 0.84g of sodium metabisulphite. Hydrolysis was

carried out by adding 50ml of 6M HCl containing 50mg of phenol to the oxidised sample, and refluxing for 24h. The hydrolysates were made up to 100ml in volumetric flasks, filtered as above, and 2ml evaporated to dryness, then stored at -20°C for subsequent analysis.

3. For tryptophan: 4.3M LiOH hydrolysis in teflon containers at 110°C for 20h. Duplicate 50 mg samples of the freeze-dried and finely ground diet, ileal digesta and faeces samples were accurately weighed into teflon containers (30ml), and 20ml of 4.3M LiOH containing 4.0µmol of 5-methyltryptophan, were added and mixed. Nitrogen gas was bubbled into the top of the container and the lid replaced and tightened. Hydrolysis proceeded at 110°C for 20h. The hydrolysates were made up to 25ml in volumetric flasks, filtered through Whatman no.3 paper with the first 3-5ml being discarded, and 7ml of the filtrate were adjusted to pH 4.5 then made up to 10ml, and stored at -20°C for subsequent analysis.

The hydrolysates were analysed on a Beckman 119 BL amino acid analyser which had been calibrated using a Beckman calibration mixture. The tryptophan values were corrected for losses during hydrolysis by 5-methyltryptophan recoveries (Chapter 2, section 2.2). Methionine and cysteine were determined as methionine sulphone and cysteic acid respectively. The diet, ileal digesta and faeces samples were corrected for loss or incomplete hydrolysis of amino acids by using the correction factors given in a previous chapter of this thesis (Chapter 2, Section 2.1, Part 2.).

All samples were analysed for total nitrogen by the Kjeldahl technique (Hiller <u>et al.</u>, 1948). The samples were weighed in duplicate, and digested in hot concentrated H_2SO_4 with mercuric sulphate as a catalyst. The ammonia formed was distilled into boric acid and subsequently titrated against standardised 0.1M H_2SO_4 using methyl red indicator.

The dry-matter content was determined in all samples. The samples were weighed in duplicate into porcelain crucibles, and dried in an

oven at 95°C to constant weight. The wet weight to freeze-dried weight ratio was already known. Also the gross energy contents of the diet and faecal samples were determined. The samples were analysed in duplicate using an adiabatic bomb calorimeter (A. Gallenkamp and Co. Ltd., Christopher St, London). The samples were pelleted before combustion.

STATISTICAL ANALYSIS.

The apparent ileal and faecal amino acid, N, dry-matter and faecal gross energy digestibilities for each subject were calculated by using equation 1.1 (section 1.3.1). The amino acid and N digestibilities were tested for homogeneity of variance around the means using Barlett's test (Snedecor and Cochran, 1982), and a model which included terms for species (pig versus human), type (ileal versus faecal), and species*type, was fitted to the data for each amino acid. Reductions in sums of squares were used to determine levels of significance.

4.3. RESULTS.

The human subjects generally completed the trial without any complications, although one subject left some food at the beginning of the period, with the amount being accurately recorded. One intact subject altered the diet after day one (from diet C to diet B), and continued on the trial for an extra day. Otherwise all subjects took care to comply with the protocol for both the diet and collection procedures.

The ileostomised pigs remained healthy after the operations and throughout the ileal collection period, with no signs of infection or illness. They had normal appetites and gained in weight (2-5kg) over the 3 weeks following surgery. The pigs ate all the food that was offered to them prior to and during both the ileal and faecal collection periods. At the end of the ileal collection period the pigs were euthanased, and an examination of the gut revealed that the large intestine had regressed in size and was paler in colour, but appeared healthy and still contained some digesta. Only one pig showed adhesions around the abdominal incision, and from parts of the small intestine to other parts of the intestine, but the stomas were healthy in all pigs.

The chemical composition of the diet is shown in Table 4.4, which compares the values determined by using food tables (Paul and Southgate, 1978) and by chemical analysis. The values shown are the only components analysed for in the present study, and they compare well with the tabulated values, except for the soluble sugars and aspartate, giving added confidence to the chemical analyses used.

After the digestibility data had been subjected to Bartlett's test, it was found that for 19 amino acids the variances were non-homogeneous, so the data for these amino acids were plotted out, and it was found that the data for apparent digestibility were consistently lower for one human ileostomate. It was found that this

Table 4.4. Comparison of the chemical composition (mg/g dry-matter) and energy content (kJ/g dry-matter) of the diet followed by twelve human subjects and twelve pigs determined by reference to food table values or by chemical analysis.

Component	Food table *1	Chemical analysis
Energy	18.6	20.5
Protein	220.5	255.7
Starch *2	252.5	255.7
Dietary fibre *2	102.1	112.3 *3
Total soluble sugars *2	264.7	187.5
Lysine	16.2	16.7
Histidine	7.7	6.0
Arginine	12.5	11.5
Aspartate	31.3	19.1
Threonine	10.4	8.6
Serine	12.7	10.8
Glutamate	44.8	46.4
Proline	16.0	15.5
Glycine	9.0	7.4
Alanine	11.4	9.7
Valine	14.1	12.0
Isoleucine	11.5	9.9
Leucine	19.4	18.2
Tyrosine	8.4	8.2
Phenylalanine	11.5	9.7
Methionine	4.8	5.1
Cysteine	7.8	9.2
Tryptophan	3.3	2.3

*1 Tabulated values from Paul and Southgate, 1978.

*2 The chemical methods used to determine the starch, dietary fibre and soluble sugar values are described in a subsequent section (Chapter 6, Section 6.3.b)

*3 The water of condensation has been subtracted from the sum of the individual sugars to give the total dietary fibre.

subject had complained of having the flu and slight diarrhoea at the beginning of the trial. Therefore it was decided to rerun the test for homogeneity of variance without this subject's data included. It was found that the variance was then homogeneous for all amino acids except proline and methionine, and N. It was also noted that when the analysis of variance was performed without this subject's data, the conclusion as to the significance of the various factors did not change greatly. It was thus considered appropriate to remove the observations for this subject from the data set.

The mean amino acid and N digestibility coefficients, are given in Table 4.5. There was no interaction for arginine, histidine, aspartate, serine, threonine, glycine, valine, leucine, tyrosine, phenylalanine, cysteine and tryptophan. There was a significant effect of digestibility type for all of these amino acids except tyrosine, and a significant effect of species for all of them. There was a significant interaction between species and digestibility type for lysine, glutamate, alanine, proline, methionine, isoleucine, and N. For these amino acids and N the effect of species was examined (ANOVA) within digestibility type and the effect of type within species, and the results are given in Table 4.6. The latter results show that there were no statistically significant differences between ileal and faecal digestibility measurements for lysine, glutamate, alanine, isoleucine and N for humans and methionine for pigs. They also show that there was a significant effect of species for methionine and isoleucine for the ileal digestibilities, and for all faecal values except lysine.

The mean dry-matter digestibilities are given in Table 4.7. There was a species*type interaction. When the analysis of variance was repeated within species for each type, and between species for each type, there was a statistically significant difference between the ileal and faecal measurements, but there was no effect of species. The mean faecal gross energy digestibilities are given in Table 4.8. There was no significant difference between the species.

Table 4.5. Mean apparent ileal and faecal amino acid and nitrogen digestibility coefficients (%), for adult humans (65kg) and growing pigs (25kg) fed a meat/vegetable/cereal/dairy product based diet.

	HUMAN		PIG		S.E[*1]	Level of significance[*2]		
Amino acid	Ileal	Faecal	Ileal	Faecal				
	(n=5)	(n=6)	(n=6)	(n=6) [*3]		S	T	S*T
Lysine	93.6	93.2	92.6	95.9	0.82	NS	NS	*
Arginine	90.2	92.6	92.1	95.7	0.47	***	***	NS
Histidine	90.2	92.2	91.9	96.7	1.15	*	**	NS
Aspartate	87.3	89.7	90.4	94.6	0.68	***	***	NS
Serine	86.7	91.8	87.5	92.9	0.73	***	***	NS
Threonine	84.7	88.8	89.3	95.7	0.60	***	***	NS
Glutamate	93.6	94.4	94.6	97.2	0.35	***	***	*
Proline	89.9	94.7	86.8	96.2	0.75	NS	***	**
Glycine	71.5	86.5	77.9	92.1	1.30	***	***	NS
Alanine	88.1	87.9	87.4	92.9	1.21	NS	*	*
Valine	89.7	90.9	91.5	94.6	0.62	***	**	NS
Isoleucine	90.9	90.7	92.8	94.9	0.49	***	*	*
Leucine	91.9	92.8	94.7	96.4	0.41	***	**	NS
Tyrosine	88.7	90.1	91.1	91.0	0.59	**	NS	NS
Phenylalanine	89.6	91.3	91.0	94.4	0.51	***	***	NS
Cysteine	85.5	90.8	82.9	87.9	1.08	**	***	NS
Methionine	93.1	83.3	90.4	91.1	0.88	**	***	***
Tryptophan	76.7	82.6	80.8	91.7	1.73	***	***	NS
Nitrogen	86.9	88.9	86.0	94.0	0.72	**	***	***

[*1] S.E. in this and all subsequent tables = standard error.

[*2] In this and all subsequent tables S = species, T = digestibility type, and S*T = species/type.

[*3] n=5 or n=6 in this and subsequent tables = 5 or 6 subjects, respectively.

Table 4.6. Levels of statistical significance for the differences between amino acid and nitrogen digestibilities analysed within species or digestibility type.

Amino acid	Effect of Type		Effect of Species	
	Human	Pig	Ileal	Faecal
Lysine	NS	***	NS	NS
Glutamate	NS	***	NS	***
Proline	**	***	NS	**
Alanine	NS	*	NS	***
Methionine	***	NS	**	***
Isoleucine	NS	***	**	***
Nitrogen	NS	***	NS	***

Table 4.7. Mean apparent ileal and faecal dry-matter digestibility coefficients (%) for adult humans (65kg) and growing pigs (25kg).

	HUMAN		PIG		S.E	Level of significance		
	Ileal	Faecal	Ileal	Faecal		S	T	S*T
	(n=5)	(n=6)	(n-6)	(n=6)				
Dry-matter	85.3	92.6	84.5	94.9	0.57	NS	***	*

Table 4.8. Mean apparent faecal gross energy digestibility coefficients (%) for adult humans (65kg) and growing pigs (25kg).

	HUMAN	PIG	S.E	Level of significance
	(n=6)	(n=6)		
Gross energy	92.6	93.5	0.59	NS

4.4. DISCUSSION.

METHODOLOGY PROBLEMS.

The method of collection of ileal digesta for the pigs, described in the methods section of this chapter was satisfactory, however several attempts had been made before the final design was achieved. The normal human collection apparatus base plate would not adhere to the pig's skin. The alternative rubber base plate was glued onto the pig firstly with Bostik glue and held with thick elastoplast around the pig. This was unsuccessful, and a second attempt using Sicomet glue also failed. The use of female pigs may be more appropriate in future work.

VARIABILITY OF RESULTS.

The results for the human apparent ileal and faecal digestibilities showed low variation between subjects, with coefficients of variation (%) ranging from 0.84 to 5.04 for glutamate and tryptophan respectively, for the ileal values, and from 0.91 to 5.13 respectively, for the faecal values. The coefficients of variation for the comparable digestibilities determined for the pig were also low. The coefficients ranged from 0.91 and 5.25 for glutamate and tryptophan respectively, for the ileal values and from 0.88 to 4.62 for the same amino acids for the faecal values.

HUMAN DIGESTIBILITY RESULTS.

The mean apparent ileal digestibility of N of 87% found in the present study for humans, suggests that the dietary protein used was almost completely digested and absorbed in the small intestine, and is similar to other values in the literature reported for humans. Russell et al. (1984) reported apparent ileal protein digestibilities of around 89% for a chemically defined meal consisting of peptides and amino acids, and for an entire protein

given to human ileostomates. Holgate and Read (1983) determined an apparent ileal protein digestibility value of 83% for meat in a single test meal, and similarly Sandberg et al. (1981) reported an apparent ileal protein digestibility for humans given a meat based diet of around 90%. Sandstrom et al. (1986) reported apparent ileal digestibilities (mean ± standard deviation) of protein for a mixed diet based on meat-protein of 79.5 ± 5.1%, while diets based on soy-protein products had slightly lower digestibility coefficients of 74.6 ± 6.2%, 74.6 ± 6.0%, and 76.8 ± 7.0% for soy-flour, soy-concentrate, and soy-isolate respectively. These latter values, although lower, are within the range recorded in the present study.

The apparent faecal digestibility of protein in human subjects has been investigated by several groups (Istfan et al., 1983; Schrimshaw et al., 1983; Wayler et al., 1983; Young et al., 1984) with meat and soy protein in particular. Istfan et al. (1983) reported apparent digestibilities of 77% to 89% for low and high soy-protein diets respectively, while Schrimshaw et al. (1983) reported apparent digestibilities for two different isolated soy-proteins of 84% and 81%, and also found that dried skim milk powder based diets had an apparent digestibility of 85%. Wayler et al. (1983) reported digestibilities for diets with different proportions of beef and soy protein of 88-89%. Young et al. (1984) reported apparent protein digestibility coefficients for isolated soy-protein and egg protein based diets, of 85 and 81% respectively. These digestibility estimates for a range of protein sources, are similar to the mean value for faecal N digestibility (89%) determined with the human subjects in the present study.

For most of the amino acids, the faecal measurement of digestibility was higher than the ileal measurement for humans. The overall mean difference was 3.7 percentage units, although for glycine, tryptophan, and proline there were differences as high as 15, 6, and 6 percentage units respectively. However, the digestibility values for methionine, lysine, alanine, and isoleucine at the ileum were

higher than those in the faeces, by 9.8, 0.4, 0.2, and 0.2 percentage units respectively. Although the differences between the ileal and faecal values were statistically significant for all but lysine, glutamate, alanine, isoleucine, tyrosine and N, the differences in practical terms were often small (less than 5%). Differences between the ileal and faecal digestibility of protein for meat and soy based diets reported in different studies with humans (Istfan et al., 1983; Schrimshaw et al., 1983; Wayler et al., 1983; Young et al., 1984; Sandstrom et al., 1986), are slightly greater (5-10%) than the difference found in the present study. Gibson et al. (1976) reported apparent ileal protein digestibilities (96% and 97%) for a low and high protein diet respectively, and in the same study compared these with apparent faecal protein digestibilities for the same diets (97% and 98% respectively). The difference of 1-2 percentage units between the ileal and faecal values for protein digestibility, is a slightly smaller difference than has been found in the present study.

The present results show that a statistically significant difference exists between amino acid digestibility values determined at the ileum or on the faeces in humans, although the difference is small in absolute terms. In the face of these small differences, if the faecal assay was more convenient, there may not be a great advantage in using the ileal assay. Given that the ileal method for determining amino acid digestibility in humans is more accurate than the faecal method for a normal meat/vegetable/cereal/dairy products-based diet, the ileal method would be even more appropriate for determining the digestibility of food ingredients that are less refined. The food ingredients that are found in developing countries, for example, are often less processed and high in dietary fibre content, and a greater difference is likely to exist between the two assays for less refined foodstuffs.

PIG DIGESTIBILITY RESULTS.

The apparent ileal and faecal protein digestibility values for the pig determined in the present study are similar to other reported values (Zebrowska, 1973b; c; Sauer et al., 1977a; b; Low, 1979a; b; 1980a; Moughan and Smith, 1985). The range of apparent ileal and faecal amino acid digestibility values for any one source are often 10 to 20 percentage units, and more (Just, 1980; Low, 1980a; Sauer and Ozimek, 1986), which is similar to the range found for the diet used in the present study.

There were significant differences between the two methods for determining digestibility for most amino acids for the pig. The only amino acids for which there were no differences between the ileal and faecal digestibilities were tyrosine and methionine. Even greater differences between the ileal and faecal digestibility determinations than those found in the present study, have been reported for pigs given less refined diets (Sauer et al., 1980). The diet used in the present study consisted mainly of proteins from milk, cheese and chicken meat, which are highly digestible, so a greater difference may be observed with more poorly digested and unrefined ingredients. The apparent faecal amino acid digestibility values have been found to be 4-5% units higher than the corresponding ileal values by Holmes et al. (1974), and on average 7.6 percentage units higher by Sauer et al. (1980). The difference between the ileal and faecal digestibility values for pigs may be up to 10-20%, especially for cysteine, threonine, tryptophan, proline, serine and glycine (Sauer et al. 1980), and the greatest differences found in the present study were also for these amino acids.

DIGESTIBILITY OF NITROGEN COMPARED WITH INDIVIDUAL AMINO ACID DIGESTIBILITY COEFFICIENTS.

The apparent ileal digestibility of dietary N in the human subjects was 87% compared to the range of amino acid values of 72-94%, although most of the amino acid values were between 86 and 91%, therefore the overall N digestibility was slightly lower than the average of the amino acid digestibilities, although only by 1-2 percentage units. The human faecal digestibility of N (89%) was also slightly lower than the average of the amino acid digestibilities, and most amino acid digestibility values were between 89 and 95%. The pig ileal N digestibility was also slightly lower than the average of the amino acid digestibilities, being 2-3 percentage units lower. The pig faecal N digestibility was the same as the average of the amino acid digestibilities. Therefore, the N digestibility values while being similar to the average of the amino acid digestibilities as would be expected, are not very useful in predicting the digestibility of protein, when the details on the individual amino acids are required. This is because the digestibility values for each amino acid differ both from the other amino acids and the overall N digestibility. The apparent ileal digestibility of N is usually similar to the average of the amino acid digestibility values (Low, 1980a), as was found for the present study. However, Mawer and Nixon (1969) suggested that the apparent absorption of amino acids would be underestimated if based on the total N absorption, due to some endogenous amino acids being present in large amounts, which resulted in low digestibilities. Nielsen (1971) has also shown that the N digestibility was lower than most of the digestibilities of amino acids, in the faeces of pigs.

DRY-MATTER AND GROSS ENERGY DIGESTIBILITIES.

The digestibility of dry-matter was significantly different when determined by the ileal or faecal methods, although the differences were smaller for humans than for pigs, being 7.4 and 10.4 percentage units respectively. There was however no effect of species, that is the ileal values were similar in the two species, as were the faecal values. The faecal digestibility of gross energy was also similar between the two species. Forsum et al. (1981) also compared the faecal energy digestibility between humans and the pig, and found good agreement, although the values for the pig were slightly lower than the human values for the two diets used in the study.

THE PIG AS A MODEL FOR HUMANS.

It is becoming increasingly difficult for ethical reasons, to carry out research in the area of human nutrition using human subjects, therefore when attempting to determine the amino acid digestibility of human foodstuffs, whether as individual ingredients, or as a mixed diet, it would be more appropriate to use a model animal to allow for routine and controlled studies. There are several animals that have been used as models in the area of clinical nutrition, however few direct comparisons have been made to establish the usefulness of animals as models for predicting the protein digestibility of human diets. Forsum et al. (1981) investigated the protein quality of two mixed diets and compared the true faecal protein digestibilities between adult humans, the growing pig (12kg) and the rat. The three species however did not receive exactly the same diets over the experimental period, and the endogenous contribution of protein was determined using different procedures. The true digestibilities for the vegetable protein diet were 80, 85 and 85%, and for the vegetable/animal protein diet were 88, 90 and 90% for the human, pig and rat respectively. Although the values were similar, Forsum et al. (1981) suggested that the use of the growing pig for evaluation of human diets should be further

investigated, because there are often practical problems with feeding rats human diets of poor protein quality. The pigs used in the latter study, however, were immature animals, being only of 12kg liveweight, and larger pigs may be better models for the adult human. The pig may also be a better model animal than the rat because the pig is a meal-eating species and does not practice coprophagy as does the rat.

Comparison of the apparent ileal digestibility values for amino acids and N between humans and the pig in the present study, shows that the difference was small, (0.7 to 6.3 percentage units for alanine and glycine respectively; Table 4.9), although statistically significant for all components except lysine, glutamate, proline, alanine and N. The pig ileal values were greater than the corresponding values in the human, except for cysteine, alanine, proline, methionine and N, which were greater in the human by an average of 1.7 percentage units. On average the difference for the digestibility of amino acids between the two species for those amino acids that had higher ileal digestibilities in the pig, was 3 percentage units. For the essential amino acids, the differences between the two species for digestibility range from 1-5 percentage units for lysine and threonine respectively.

Comparison of the apparent faecal digestibilities between the two species also showed differences for all amino acids except lysine, and N, although the differences were small. This may be caused by the differences in relative hind-gut volumes of the two species (see Table 1.1). The faecal method for determining the digestibility of amino acids may still be useful for application to human foodstuffs, especially if the ingredients are refined and highly digestible, because in these cases the ileal/ faecal differences are likely to be small. The ileal/faecal differences for each species in the present study were similar although slightly greater for the pig. In absolute terms, there was on average, a greater magnitude of difference between the ileal and faecal measurements (approximately

Table 4.9. The differences[*1] for ileal and faecal amino acid and nitrogen digestibility coefficients between humans and pigs.

Amino acid	Ileal	Faecal
Lysine	1.0	2.7
Arginine	1.9	3.1
Histidine	1.7	4.5
Aspartate	3.1	4.9
Serine	0.8	1.1
Threonine	4.6	6.9
Glutamate	1.0	2.8
Proline	3.1	1.5
Glycine	6.4	5.6
Alanine	0.7	5.0
Valine	1.8	3.7
Isoleucine	1.9	4.2
Leucine	2.8	3.6
Tyrosine	2.4	0.9
Phenylalanine	1.4	3.1
Cysteine	2.6	2.9
Methionine	2.6	7.8
Tryptophan	4.1	9.1
Nitrogen	0.9	5.1

[*1] Absolute differences between species.

4.5 percentage units), for the pig compared to humans. The faecal method in the pig is easier to conduct as it does not require surgical modification or slaughter of the animals, and is also likely to be less expensive. Forsum et al. (1981) also found that the true faecal digestibility of N was greater for the pig than the human, and that the difference (5 percentage units) was greater for a diet containing only vegetable protein than the difference (1.5 percentage units) for a diet containing both vegetable and animal protein.

ILEOSTOMY MODEL.

The ileostomised pig was used in this study as a model for the ileostomised person, however it is possible that the ileostomised person does not represent a normal situation for determining the ileal amino acid digestibility in humans, due to microbial colonisation of the terminal ileum following surgery. It was assumed that the colonisation of the pig ileum following surgery would be as extensive as that in humans, as the microflora are reported to become stable 1-3 weeks following surgery (Vince et al., 1973). However, if the effect of the microflora in humans was significant enough to affect the determination of amino acid digestibility, it is likely that the determined digestibility values would tend to be higher than those in the normal human small intestine, and therefore the ileal/faecal differences found in the present study may be even greater in reality.

ALTERNATIVE METHODS OF COLLECTION.

If the pig was to be used as a model for humans for apparent ileal amino acid digestibility determination, there are other methods used for the collection of ileal digesta that may be better than the formation of an ileostomy, and so giving values that are closer to those that may be obtained if normal intact human subjects could be used. Several other methods are commonly used in digestibility

studies with pigs to allow for collection of digesta at the terminal ileum, and these all have their advantages and disadvantages. The easiest of these methods involves slaughter of the animals with collection of the gut contents. However, some methods of slaughter have been shown to cause mucosal cell shedding (Low, 1980a; Rerat, 1981), so often the animals are anaesthetised before removing the gut, and the animals are then killed using barbiturates (Low, 1980a). This method, however, only allows one measurement to be obtained from each pig. There are also various surgical techniques that are commonly used, though these are more complex. Also these may lead to a physiologically abnormal situation as the modification to the gut may result in incomplete innervation or abnormal gut secretions in the region of the gut that has been modified. These cannulation methods do however allow much more information to be gathered from one pig as the animal may be used for collection over a period of time and repeated observations may be performed (Low 1980a).

The most commonly used surgical method for the collection of terminal ileal digesta is simple T-piece cannulation, which allows discontinuous sampling to be carried out. The cannula is usually placed 5-10cm before the ileocaecal valve, and this method avoids transection of the intestine which interrupts the migration of the myoelectric complexes in the gut (Sauer and Ozimek, 1986), although the sampling of digesta may not be representative, and frequent sampling is required to avoid this potential problem. The re-entrant cannula avoids the problem of non-representative sampling, as the entire flow of digesta is diverted through an exit cannula so that sampling can take place, then the remainder of the digesta is returned to the gut via an entry cannula (Low, 1980a). This method does, however, involve transection of the gut, and there can be problems of blockage of the cannulas, depending on the nature of the diet used (Sauer and Ozimek, 1986). The surgery involved is also more complex than that for the T-cannula, and the survival of the animals poorer (Low, 1980a). Several comparisons of digestibility

obtained by using either of these cannulation methods have shown that the two methods generally give similar results (Zebrowska et al., 1978). The effect of cannulation on the overall rate of passage and digestibility of organic matter, crude-protein, N-free output, and amino acids is generally small (Sauer and Aherne, 1979; Sauer and Ozimek, 1986). Another method for the total collection of digesta is the ileocolic post-valvular procedure (Darcy et al., 1980), which preserves the functional role of the ileocaecal valve. The use of ileorectal anastomosis (Fuller and Livingstone, 1982) has also been used for the total collection of ileal digesta, with the end of the ileum being fitted to the side of the colon just before the rectum. The amino acid digestibility values obtained using this method are similar to those obtained using ileocaecal re-entrant cannulas (Sauer and Ozimek, 1986).

As mentioned earlier, the present study could be criticised due to the human ileal digestibility determinations being made using the ileostomate, which may not simulate normal conditions. Also, the ileostomates used in the study all had well-established ileostomies, while the ileostomised pigs used for comparison with the human subjects only had the operations 2-3 weeks before the trial took place. Vince et al. (1973) have reported, however, that a characteristic microflora becomes established 1-3 weeks after an ileostomy in human subjects. It is very difficult to establish completely valid comparison between two different species of animals, and the comparative results must, therefore, always be treated cautiously. Some measurements on the level of microbial activity in the terminal ileum of each species were made and are reported in Chapter 6. The latter measures provide some information on the acceptability of comparing digestion in ileostomised pigs and humans.

CHAPTER 5.

DETERMINATION OF ENDOGENOUS AMINO ACIDS AT THE TERMINAL ILEUM OF THE ADULT HUMAN AND GROWING PIG, AND COMPARISON BETWEEN THE TWO SPECIES OF THE TRUE ILEAL DIGESTIBILITY OF PROTEIN AND AMINO ACIDS IN A MIXED DIET.

5.1. INTRODUCTION.

The true ileal digestibility of amino acids takes into account the endogenous contribution of amino acids at the end of the small intestine from digestive enzymes, mucosal cells, mucoproteins and other nitrogenous compounds found in gut secretions. When investigating the use of the pig as a model animal for the human for ileal amino acid digestibility estimation, and so comparing digestibility values between the two species, it may be important to use true digestibility values. The endogenous contributions of amino acids may be different in the two species, and so true digestibilities may give a more accurate basis for comparison.

The endogenous contribution is often determined by collecting digesta, following the consumption of a protein-free diet for several days, although in work with humans it is more acceptable to the subjects to feed a single meal only, and collect the ileal output over several hours. The rate of gastric emptying of a liquid meal in humans has been determined to be around 2h (Hunt and Spurrell, 1951), and of a semi-solid meal to be approximately 4h (Malagelada et al., 1976). The transit of material through the small intestine is 1-3h (Dillard et al., 1965), and so most of an average sized meal should have reached the terminal ileum within 4-8h after ingestion (McMinn and Hobdell, 1974; Holgate and Read, 1983; Magee and Dalley, 1986). The digesta must only represent that associated with the protein-free meal, therefore it is important to ensure that there will be no contamination due to food consumed prior to the study. The easiest and most acceptable way to ensure this, is for

the subject to fast to clear the gut of any residual digesta. Following the consumption of a meal, most of it will have reached the terminal ileum after about 6h, and therefore a fasting period of 14h, which is acceptable to human subjects, should be sufficient to clear the stomach and small intestine of digesta. This fasting period can be achieved overnight, and so is convenient for the subjects involved.

The protein-free meal must consist of non-protein containing ingredients only, and the level of fibre included should be similar to that in the diet that was used for collection of digesta for which the true digestibility values are being determined. The level and type of fibre may influence the endogenous contributions of amino acids (Sauer and Ozimek, 1986). In the present study, the level of dry-matter consumption used was approximately the amount eaten over 8h by the subjects in the main study, described in a previous chapter (Chapter 4), but purified cellulose was the only fibre component included.

The collection of digesta over a short period of time requires the use of a non-digestible marker, and as acid-insoluble ash (as Celite) has been shown to be suitable for use in human studies (refer Chapter 3), this was used as a marker in the present study. The aim of the present study was to determine the endogenous flows of amino acids in the adult human and the growing pig, and to use these flows to correct the apparent ileal digestibilities reported in Chapter 4, giving true coefficients to allow a more refined comparison to be made between the two species.

5.2. METHODS AND MATERIALS.

PROCEDURE.

Ethics approval to conduct this study was obtained from the Massey University Human Ethics Committee, before the onset of the trial and all subjects gave their written consent with full knowledge of the experimental procedure. Six healthy adult Caucasian volunteers (two men and four women; ages 35-60 years) with well-established ileostomies (> one year), were requested to fast for 14h (over-night). This length of time was considered acceptable to ensure complete emptying of the stomach and small intestine. The subjects were not those involved in the main study (Section 4.2), and had undergone surgery due to ulcerative colitis.

Six healthy 28kg liveweight entire male pigs (Landrace x (Landrace x Large White)) with ileostomies (see Section 4.2), were kept singly in smooth-walled metabolism cages and were housed at a temperature of 22 $\pm$ 1°C. They were also subjected to a 14h fasting period to ensure that the stomach and small intestine were empty.

For both the humans and pigs, a fresh ileostomy bag was fastened after the 14h fast. At the same time, a single protein-free meal (Table 5.1) consisting of 240g$\pm$1g of a specially prepared biscuit, baked as two batches, one for the human study and one for the pig study, was consumed during the next half an hour. The amount of biscuit allowed was approximately equal to the amount of dry-matter eaten over 8h by the humans and pigs in the main study (approximately 200g, diet C; Section 4.2). All subjects were allowed to consume unlimited quantities of black tea or coffee (with sugar), water and lemonade, and the pigs received water *ad libitum*, during the 8h following the commencement of the study period, during which time all ileostomy output was collected. The ileostomy bags were emptied on the 3rd, 6th and 8th hours, and the contents placed in a plastic bag containing 10ml of 40% hydrochloric acid (HCl). The

Table 5.1. The ingredient composition of protein-free biscuits[*1] fed to ileostomised adult humans (65kg) and growing pigs (28kg) to determine the endogenous flows of amino acids at the terminal ileum.

Ingredient	Composition (g/100gdry-matter)
Cornflour[*2]	44.0
Margarine[*3]	28.5
Sugar	16.5
Golden syrup[*4]	6.6
Cellulose[*5]	2.7
Salt	1.0
Celite[*6]	0.5
Ground ginger	0.2

[*1] The biscuits contained 0.11g total nitrogen per 100g dry-matter.

[*2] Feilders Cornflour, Bluebird Foods Ltd., Auckland.

[*3] Meadow-lea table margarine, Aspak Industries Ltd., Auckland.

[*4] Chelsea Golden syrup, New Zealand Sugar Co. Ltd., Auckland.

[*5] Avicel, microcrystalline form of cellulose.

[*6] Double-acid washed celite, (Sigma Chemical Co., U.S.A.) added as an indigestible marker compound.

digesta were kept on ice during the 8h, after which the bags and contents were weighed and immediately frozen. All of the digesta samples and samples of the meal consumed were freeze-dried, weighed, finely ground (1mm), and frozen for subsequent analysis.

CHEMICAL ANALYSIS.

Dry-matter, nitrogen and amino acid analyses were carried out as described in Section 4.2, and the analysis for acid-insoluble ash was carried out as described in Section 3.2.

STATISTICAL ANALYSIS.

The endogenous flows of amino acids and N at the ileum were calculated according to equation 1.3 (Section 1.3.1). These were expressed in units of mg/24h, assuming three equal sized meals consumed daily 8h apart, rather than relative to food dry-matter intake. This is because at low levels of dry-matter intake (as in the present study), there may not be a linear relationship between endogenous loss and dry-matter intake. The flows were used to calculate true digestibility coefficients (for the data given in Section 4.3) using equation 1.2 (refer Section 1.3.1). The data were subjected to a one-way analysis of variance (Snedecor and Cochran, 1982).

5.3. RESULTS.

The protein-free meal was eaten quickly by the pigs, however the human subjects took the full half hour to consume the meal. There was very little digesta collected within the first 3h, with most of the digesta being collected between 3 and 6h. There was on average approximately twice as much material collected over the 8h period from the pigs as from the human subjects, with mean (± S.E.) wet- and dry-matter outputs in the humans of 116.5g (32.67g) and 9.1g (3.51g) respectively, and in the pigs of 158.2g (19.24g) and 16.2g (1.67) respectively. Over the total 8h period of digesta collection, 40 and 75% of the ingested acid-insoluble ash was recovered in the humans and pigs respectively. The concentration of the marker in the digesta was similar for both species.

The mean ileal endogenous amino acid and N flows for the adult human and growing pig are given in Table 5.2. The ileal flows of amino acids and N in the human were generally greater than for the pig, and there were significant differences between the two species for all amino acids except proline, alanine, methionine and N.

The mean true ileal amino acid and N digestibility coefficients for the adult human and growing pig fed the diet described in Section 4.2, determined using the endogenous amino acid flows shown here, are given in Table 5.3. There were no significant differences between the two species for the digestibility values for most amino acids. For those amino acids for which there was a significant difference between the species (threonine, phenylalanine, methionine and cysteine), the actual differences were small, being less than 2.4% units for all but cysteine and threonine which differed by 8 and 5% units respectively.

Table 5.2. Mean endogenous ileal flows of amino acids (mg/day) in adult humans (65kg) and the growing pig (28kg) following a single protein-free meal.

Amino acid	Human (n=6)	Pig (n=6)	S.E	Level of significance
Lysine	661.7	394.7	80.04	**
Histidine	343.3	162.8	46.49	***
Arginine	511.7	354.1	47.61	*
Aspartate	1216.9	757.1	147.91	**
Serine	906.4	567.9	104.66	*
Threonine	1114.1	593.0	139.69	**
Glutamate	1440.6	892.1	168.16	*
Proline	1075.8	1075.5	108.72	NS
Glycine	864.4	509.6	105.11	**
Alanine	656.7	485.1	67.56	NS
Valine	825.4	461.3	106.98	**
Isoleucine	493.9	297.8	57.28	**
Leucine	937.3	604.2	107.73	*
Tyrosine	542.5	307.1	73.10	*
Phenylalanine	634.2	327.6	105.02	*
Tryptophan	350.6	196.6	54.15	*
Cysteine	858.7	487.1	130.81	*
Methionine	166.5	144.9	13.69	NS
Nitrogen	2800.3	2179.5	381.09	NS

Table 5.3. <u>The mean true ileal amino acid and nitrogen digestibilities (%) for adult humans (65kg) and the growing pig (28kg) given a meat/vegetable/cereal/dairy product based diet.</u>

Amino acid	Human (n=6)	Pig (n=6)	S.E	Level of significance
Lysine	98.7	97.5	0.41	NS
Histidine	99.2	97.5	0.65	NS
Arginine	98.3	97.7	0.48	NS
Aspartate	99.2	97.8	0.61	NS
Serine	99.4	99.5	0.61	NS
Threonine	105.1	99.7	1.36	**
Glutamate	98.5	98.2	0.34	NS
Proline	100.9	98.1	1.13	NS
Glycine	92.4	90.5	1.28	NS
Alanine	98.9	98.0	0.48	NS
Valine	99.5	98.6	0.53	NS
Isoleucine	99.5	97.6	0.64	NS
Leucine	100.1	100.8	0.38	NS
Tyrosine	99.2	98.0	0.51	NS
Phenylalanine	99.9	97.5	0.69	*
Tryptophan	99.1	96.8	2.33	NS
Cysteine	100.3	92.2	1.95	***
Methionine	98.4	96.0	0.64	**
Nitrogen	98.0	98.7	1.17	NS

5.4. DISCUSSION.

The low recovery of Celite (40%) in humans compared with the pig (75%) was surprising considering that the stomach emptying and small intestinal transit rates were expected to be similar for the two species (refer Sections 1.4.1 and 1.4.2). The differences may have occurred due to the unusual nature of the diet used in the present study. In both species, however, a reasonable proportion of the total diet-associated flow was collected, which means that representative endogenous flow values should have been obtained.

The mean endogenous flows of amino acids and N were highly variable, with coefficients of variation ranging from 21.5 to 53.6% for methionine and phenylalanine respectively. The human ileal endogenous flows of amino acids determined in the present study were higher than the values reported by Nixon and Mawer (1970b) calculated from data for two human subjects following a similar essentially protein-free single meal. The digesta collected in the latter study were obtained from the upper ileum by intubation of the subjects after they had consumed a meal of only around 100g dry-matter. Both the site and method of collection, and the small meal size in comparison with the present study, may explain the differences. Chacko and Cummings (1988) determined the obligatory N losses from the human small intestine using ileostomy subjects, as being approximately 1g/day, which is also lower than the value found in the present study. The pig ileal endogenous flows of amino acids determined in the present study were also higher than values reported in the literature for pigs fed similar diets (Sauer et al., 1977b; Zebrowska et al., 1982) over a number of days. The collection of digesta following ingestion of a single protein-free meal does not allow the gut to adapt to the protein-free state, and the flows of endogenous amino acids may be lower after the subject has been eating a protein-free diet for several days than following a single meal. Adaptation of the gut to the protein-free state may lead to underestimation of the endogenous loss of protein. This may explain

why the flows determined in the present study for the pig appear to be high, and so may be the human flows. Another reason why the endogenous flows determined in the present study could be high is that there may be some diurnal effect on protein loss in the gut. In the present study the digesta were collected for 8h during the day, and the daily flows were estimated by assuming this represented one third of a day, when in fact a total 24h period which includes night-time may give lower results.

The mean endogenous flows of amino acids and N were quite different between the species with the human values generally higher than the pig values. There were statistically significant differences between the species for all of the amino acids except proline, alanine and methionine and N. It is not surprising that the two species have different endogenous amino acid flows, as the adult human and 28kg pig have quite different body weights and gut sizes relative to body size. The total flows were similar for the two species if expressed on the basis of bodyweight, however, this is probably by chance as the relative gut sizes are expected to be quite different. Although the absolute flows of endogenous amino acids may differ between the two species because of differences in gut size per unit body weight, it is possible that the composition of the flow would be less influenced. It is thus appropriate to compare the composition of endogenous amino acid flows between the two species. The relative molar concentrations of amino acids in the endogenous material were calculated, and are compared between species in Table 5.4. There were no significant differences between the two species for most amino acids except histidine, threonine, alanine, valine, and methionine. It is concluded, therefore, that the relative proportions of amino acids present in the endogenous material are similar between the two species. This suggests that the relative secretion of various endogenous factors is similar between humans and pigs.

Table 5.4. The mean amino acid composition (%*1) of the endogenous protein at the terminal ileum of the 28kg pig and adult human (65kg) after consumption of a single protein-free meal.

Amino acid	Human (n=6)	Pig (n=6)	S.E.	Level of significance
Lysine	4.8	4.6	0.32	NS
Histidine	2.5	1.9	0.14	**
Arginine	3.7	4.1	0.30	NS
Aspartate	9.2	8.8	0.19	NS
Serine	6.6	6.6	0.16	NS
Threonine	8.2	6.9	0.27	**
Glutamate	10.5	10.4	0.28	NS
Proline	7.9	12.5	1.18	NS
Glycine	6.3	5.9	0.32	NS
Alanine	4.8	5.6	0.12	***
Valine	6.1	5.4	0.26	*
Isoleucine	3.6	3.5	0.10	NS
Leucine	6.9	7.0	0.22	NS
Tyrosine	4.0	3.6	0.22	NS
Phenylalanine	4.6	3.8	0.42	NS
Tryptophan	2.6	2.3	0.16	NS
Cysteine	6.3	5.7	0.58	NS
Methionine	1.2	1.7	0.11	*

*1 Each amino acid expressed as a proportion of the total amino acids.

The true digestibility values were similar between the two species although the human values were a little higher than the pig values for all amino acids except leucine and for N. The digestibility values were all high, and for the human values these were usually around 98-100%, and the pig values around 97-99%. This reflects the highly digestible nature of the diet used, in which the main protein sources consisted of chicken meat, cheese, milk and refined cereal proteins, and therefore digestion and absorption may have been completed by the end of the ileum. Very few of the true coefficients were greater than 100%.

The effect of species on ileal digestibility was not as great when true coefficients were used as it was when apparent values were determined (refer Chapter 4). There was a significant difference between the two species for 13 of the 18 amino acids in the case of the apparent digestibility coefficients, whereas there were only significant differences between the species for four amino acids for the true coefficients. The differences between the species for the apparent and true amino acid digestibility determinations respectively, are given in Table 5.5. For most of the amino acids and for N, the differences between the species were smaller for the true digestibility coefficients than for the apparent values. It appears, therefore, that when comparing two species, as was an aim of the present study, the use of true digestibility determinations is superior.

The true ileal digestibilities found for human subjects in the present study are higher than values of true protein digestibility reported by other workers, and this may be due to different methods used to estimate the endogenous contribution of protein or amino acids in the gut or to the high digestibility of the diet. Sandstrom et al. (1986) reported true ileal digestibilities of protein in meat-based and soy-based diets of 85 and 80% respectively, using values for obligatory faecal loss of 8.8mg N/kg body weight. The obligatory ileal loss found in the present study is 3-6 times higher

Table 5.5. The differences[*1] between the adult human (65kg) and the growing pig (28kg) for the apparent and true ileal amino acid digestibility coefficients of a mixed diet.

Amino acid	Apparent	True
Lysine	1.0	1.2
Histidine	1.9	1.7
Arginine	1.7	0.7
Aspartate	3.1	1.4
Threonine	4.6	5.4
Serine	0.8	0.3
Glutamate	1.0	0.3
Proline	3.1	2.8
Glycine	6.9	1.9
Alanine	1.8	0.9
Cysteine	2.6	8.1
Valine	1.8	0.9
Methionine	2.7	2.4
Isoleucine	1.9	1.9
Leucine	2.8	0.7
Tyrosine	2.4	1.2
Phenylalanine	1.4	2.4
Tryptophan	4.1	2.3
Nitrogen	0.9	0.7

[*1] The absolute difference expressed as percentage units.

than the latter value, and therefore, the actual true digestibilities calculated in the study by Sandstrom et al. (1986) may be higher than reported. The results would then be closer to the true digestibilities determined in the present study. Chacko and Cummings (1988) determined true ileal N digestibilities with human ileostomy subjects, and reported values of 73-90% for a range of diets, including a value of 88.5% for a diet similar to that used in the present study, although again the endogenous N value was lower than that used to determine the true coefficients in the present study.

In studies involving human subjects, the protein-free method for determining endogenous protein loss is the most common procedure. In work using pigs and rats, however, it has recently been shown that this approach may lead to error (Darragh et al., 1989; De lange, 1989; Moughan and Rutherfurd, in prep.). The feeding of single meals may also be criticised, as the fasting period required prior to the meal may not be sufficient to completely clear the digestive tract as the stimulus of eating may aid digesta passage, and it is physiologically abnormal to eat protein-free meals. Also, in humans, the secretion of pancreatic enzymes has been shown to be stimulated by dietary protein (Roy et al., 1967), and therefore the use of protein-free diets to measure endogenous loss could lead to an underestimate. The protein-free diet used in the present study included cellulose, which is a purified fibre and was quite different to the fibre component of the diet for which the digestibility coefficients were determined. This is also likely to have an effect on the flows of endogenous amino acids in the small intestine. The true digestibility coefficients calculated using the endogenous flows determined in the present study should therefore be interpreted with caution, but are probably an improvement over apparent estimates of digestibility. In conclusion, it appears that for the diet used in the present study, the digestion of protein and absorption of amino acids was virtually complete by the end of the small intestine. The presently reported true digestibility

coefficients, however, may be overestimates and it is probable that the actual amino acid digestibilities of the diet fall somewhere between the true and apparent coefficients determined in the present study. Overall, the pig seems to be a suitable model for the adult human for determining true ileal amino acid and N digestibility.

Chapter 6.

THE USE OF BACTERIAL MARKERS TO INDICATE THE LEVELS OF BACTERIA PRESENT AND THE EXTENT OF BACTERIAL ACTIVITY IN ILEAL DIGESTA AND FAECES SAMPLES.

GENERAL INTRODUCTION.

In Chapter 4 a study was described which involved determining ileal protein digestibility, after the collection of ileal digesta from ileostomised humans and pigs. It is known, however, that in the ileostomate there is a certain degree of colonisation of the lower small intestine with bacteria and other microorganisms (Vince et al., 1973). It is not known to what extent this presence of a microflorum affects the measures of protein digestibility. The aim of the study reported in this chapter was to ascertain the significance of the small intestinal microbial metabolism in the ileostomised human and pig.

It would be expected that levels of bacteria would be much higher in the faeces than in ileal digesta (Williams Smith, 1965; Drasar and Hill, 1974; Drasar and Barrow, 1985). Thus, comparison of the levels of bacteria and the extent of microbial activity, in faeces and ileal digesta, may provide useful information on the degree of colonisation of the ileum following an ileostomy operation. In the present work the levels of bacteria were determined by reference to bacterial markers, compounds which can be used to determine the presence of bacteria, and the extent of bacterial activity was examined by determining the degree of breakdown of dietary fibre components.

There are several substances commonly used as bacterial markers, however the different markers may not be consistent for determining the levels of bacteria present (Siddons et al., 1982; Ushida et al., 1985). This study used two markers (nucleic acids and diaminopimelic

acid) for determining the levels of bacteria present in diet, ileal digesta and faeces samples.

SECTION 6.1. THE USE OF NUCLEIC ACIDS TO INDICATE THE LEVELS OF BACTERIA PRESENT IN DIET, ILEAL DIGESTA AND FAECES SAMPLES.

a. INTRODUCTION.

The measurement of nucleic acids in feed and digesta samples has been carried out for many years in ruminant studies to indicate the amount of microbial material present in samples. It was first used by Gausseres and Fauconneau (1965), who measured DNA and RNA on their samples by fractional separation of RNA mononucleotides, and DNA purine bases. Their method has been criticised as being time-consuming and unsuitable for routine use. Several workers have since suggested ways to improve the method for measuring DNA and RNA, and a series of studies (McAllan and Smith, 1969; 1972; 1973; 1983; 1984) has investigated the method, and the advantages and disadvantages of using DNA and RNA measurements as bacterial markers. Some of the potential problems that may exist when using nucleic acids as bacterial markers include the possibility of dietary nucleic acids avoiding digestion in the gut, although McAllan and Smith (1972) have shown that exogenous DNA and RNA are rapidly degraded in the rumen. Also, the nucleic acid levels in shed mucosal cells in the gut may lead to overestimation of the bacterial cells present.

Hutchison and Munro (1961) give a detailed description of the main methods used for nucleic acid determination and the possible errors that can be introduced. They recommend the method of Schmidt and Thannhauser (1945) as being a good base method, although there may be problems when the method is applied to plant tissue and bacterial samples. Munro and Fleck (1969), also recommend using the Schmidt-Thannhauser method, and they describe a modification of the method based on the observations of other workers. They also suggest that the conditions be examined in each situation for the determination of nucleic acid content in unknown samples.

After reviewing the literature, and carring out some preliminary work, the Schmidt-Thannhauser method was adopted in the present study, and modifications were made according to the suggestions of different workers (Ogur and Rosen, 1950; Wyatt, 1951; Smillie and Krotkov, 1960; Hutchison and Munro, 1961; Hutchison et al., 1962; McAllan and Smith, 1969; Munro and Fleck, 1969). This method involves digestion of the sample with dilute alkali, which hydrolyses RNA and renders it soluble in acid, but DNA is resistant to attack by the alkali. The DNA is then extracted with hot acid from the precipitate obtained after alkaline digestion.

There are many methods available for measuring the RNA and DNA contents of the respective fractions, the most commonly used being ultraviolet absorption at 260nm or the colour reaction with orcinol for RNA (Bial, 1902, cited by Munro and Fleck 1969), and diphenylamine for DNA (Burton, 1956). The orcinol reaction for the determination of RNA, has been modified over the years, and the present study will use a method based on these modifications (Massart and Hoste, 1947; Lin and Schjeide, 1969). The diphenylamine reaction has also been modified over the years (Burton, 1956; Giles and Myers, 1965). Abraham et al. (1972) have shown that by including an additional organic extraction using amyl acetate after colour development, a four fold increase in the sensitivity can be obtained. This is particularly useful when analysing samples with low DNA content, and for samples which would otherwise be turbid after colour development. The method described by Giles and Myers (1965) with the additional step of Abraham et al. (1972) was used for the detection of DNA in the present study.

The present study aimed to determine the levels of DNA and RNA in human and pig ileal digesta and faeces samples. A second aim was to compare the detection of RNA in the respective fractions by ultraviolet absorption and the orcinol reaction.

b. METHODS AND MATERIALS.

REAGENTS.

All reagents used were of AnalaR quality.
Perchloric acid (PCA), BDH, England.
Ethanol, BDH, England.
Diethyl ether (anhydrous), May and Baker Ltd., Australia.
Chloroform, Ajax Chemical Co., Australia.
Potassium Hydroxide (KOH), May and Baker Ltd., Australia.
Hydrochloric acid (HCl), BDH, England.
Tris(hydroxymethyl)methyl amine buffer grade, (Tris), U.S. Biochemical Corp., U.S.A.
Dowex-1 resin (1x2-100), Sigma Chemical Co., U.S.A.
Orcinol: 5g orcinol (BDH, England) dissolved in 10 ml ethanol, stored at 5°C.
Copper (II) chloride ($CuCl_2$) solution: 0.25g $CuCl_2$ (Ajax Chemicals, Australia) dissolved in 165ml concentrated HCl, stored at room temperature.
Amyl acetate, May and Baker Ltd., Australia.
Acetaldehyde: 0.16g acetaldehyde (Merck-Schuchardt, Germany) made up to 100 ml with cold dH_2O.
Diphenylamine solution: 40g of diphenylamine (May and Baker Ltd., Australia) dissolved in 1l of glacial acetic acid (Ajax Chemicals, Australia).
DNA standard, Sigma Chemical Co., U.S.A.
RNA standard, (purified bovine mammary RNA, P.Mead, Massey Univ., N.Z.)

CHEMICAL ANALYSIS.

1.LIPID EXTRACTION. Duplicate 2g samples of a freeze-dried diet, ileal digesta or faeces sample, collected as described in Chapter 4, were accurately weighed in duplicate into preweighed centrifuge tubes, and kept on ice. Ten ml of cold absolute ethanol were added

to the tubes and mixed well, then centrifuged at 35000xg for 15min. The supernatant was decanted off and discarded, and the extraction was repeated with a second 10ml of ethanol. The extraction was then repeated with 10ml of a 1:3 chloroform/ ethanol mixture, followed by 10ml of diethyl ether. The supernatant was decanted off and discarded, and the residue allowed to dry at air temperature. The tube was then reweighed, the duplicates pooled and the residue finely ground for subsequent analysis.

2.FRACTIONATION OF NUCLEIC ACIDS. Duplicate 0.5g samples of the extracted material were accurately weighed into centrifuge tubes, and 10ml of cold 0.2M PCA were added and mixed, then allowed to stand on ice for 10min. The tubes were centrifuged for 5min at 10000xg, and the supernatant decanted off and discarded. The residue was washed twice by resuspending it in 10ml of 0.2M PCA and recentrifuging, with as much acid removed as possible on the last time. Five ml of 0.3M KOH were added, mixed, and the tubes incubated at 37°C for 20h. After the incubation, the tubes were cooled on ice and 3.0ml of 1.25M PCA were added. The tubes were centrifuged at 10000xg for 5min, and the supernatant decanted off and collected in a 50ml volumetric flask. The residue was washed twice with 2x5ml of 0.25M PCA and the washings added to the volumetric flask, which was made up to volume and stored at -20°C for subsequent RNA analysis.

The residue was resuspended in 10ml of 1.0M PCA, and incubated at 70°C for 1h. After the incubation the tubes were cooled, and centrifuged at 10000xg for 5min, and the supernatant decanted off and collected in a 50ml volumetric flask. The residue was washed twice with 2x5ml of 1M PCA, and the washings were added to the volumetric flask, which was made up to volume and stored at -20°C for subsequent DNA analysis.

3.RNA ANALYSIS. One g of wet Dowex-1 ion exchange resin was washed into a 6ml Pierce disposable polystyrene column, and washed with loading buffer (0.025M-tris, pH 7.8), to give the dimensions of the

resin bed of 3.0cmx0.5cm. One ml of the RNA fraction was adjusted to pH 7, and made up to a volume of 5ml with the loading buffer. This was then loaded onto the column, the 25ml of eluate collected by eluting with 0.5M HCl. The eluate was stored at -20°C for subsequent analysis.

After making any necessary dilutions, 2ml of the RNA sample were mixed with 2ml of the orcinol:$CuCl_2$ reagent, and placed in a boiling water bath for 37mins. The tubes were cooled and the absorbance read at 666nm. A standard RNA curve was also plotted. The absorbance of the RNA sample was also measured at 260 nm, and a standard curve was prepared.

4. DNA ANALYSIS. The DNA fraction was also diluted if necessary, and 2ml of the fraction were mixed with 2ml of fresh diphenylamine solution and 0.1ml of fresh acetaldehyde solution. The tubes were incubated at 30°C for 16-20h. After the tubes had cooled, 1ml of amyl acetate was added, mixed, and allowed to sit while the separate layers formed. The top layer was carefully removed with a Pasteur pipette, and its absorbance read at 595nm. A standard curve was also prepared.

5. All unknown samples were compared against the appropriate standard curves and the amount of RNA-N and DNA-N were determined for each sample assuming that RNA and DNA contain 14.8 and 14.0% N respectively (Ling and Buttery, 1978).

STATISTICAL ANALYSIS.

The data were tested for homogeneity of variance around the means using Barlett's test (Snedecor and Cochran, 1982), and a model which included terms for species (human versus pig), type (ileal versus faecal), and species*type, was fitted to the data. Reductions in the sums of squares were used to determine levels of significance.

c. RESULTS.

The diet samples had low DNA and RNA contents (2 and 4mg/g dry-matter respectively) compared with the other samples. The mean results for the RNA and DNA analyses for the ileal digesta and faeces samples are given in Tables 6.1 and 6.2 respectively. Table 6.2 also includes a comparison of the two methods of detection for RNA. The results from the two methods for the determination of RNA did not agree for the ileal digesta, although the faeces values were in closer agreement. The orcinol method gave higher RNA values for all samples except the human faeces, which was higher when determined by the ultraviolet absorption method. The RNA values were highly variable between individuals, and the agreement between duplicates was poor (up to 20% difference expressed as a proportion of the mean), so these data were not analysed statistically.

The results of the DNA determination when expressed as the ratio of the amount of DNA-N to total N in each sample showed that the faeces samples had a higher concentration of DNA than the ileal digesta for both species. When the DNA-N was expressed as the total output per day, the human faecal value was still greater than the ileal value, however for the pig, the ileal value was higher than the faecal value.

There was a significant species*type interaction for the amount of DNA-N excreted per day, therefore an analysis of variance was performed using these data within species for type, and within type for species. There was a significant difference between pig ileal and faecal values ($P<0.01$), between human ileal and faecal samples ($P<0.05$), and between human and pig faecal values ($P<0.001$), but no significant difference between human and pig ileal samples.

Table 6.1. The mean RNA concentrations (mg RNA-N:g Total-N) in ileal digesta and faeces samples collected from adult humans (65kg) and growing pigs (25kg), and the mean daily output of RNA (mg RNA-N/day).

	HUMAN		PIG	
	Ileal (n=5)	Faecal (n=6)	Ileal (n=5)	Faecal (n=6)
RNA-N:Total-N in sample				
Orcinol method	114.9	42.0	57.5	32.3
A_{260} method	32.5	51.7	27.4	23.0
RNA-N mg/day				
Orcinol method	274.7	68.5	184.3	36.7
A_{260} method	77.7	84.3	87.8	26.1

Table 6.2. The mean DNA concentration (mg DNA-N:g Total-N) in ileal digesta and faeces samples collected from adult humans (65kg) and growing pigs (25kg), and the mean daily output of DNA (mg DNA-N/day).

	HUMAN		PIG		S.E	Level of significance		
	Ileal (n=5)	Faecal (n=6)	Ileal (n=6)	Faecal (n=6)		S	T	S*T
DNA-N:Total-N in sample	4.1	8.1	4.4	5.9	0.75	NS	**	NS
DNA-N mg/day	9.8	13.3	14.1	6.8	2.14	**	*	**

d. DISCUSSION.

The RNA values did not agree well with the trend observed for the DNA values, and the two methods gave contradictory trends for the human values. The human ileal values were greater than the faecal values by the orcinol reaction, but smaller by the 260nm method. The pig ileal values appear to be similar to the pig faecal values for the 260nm method, although the orcinol determination again gave higher ileal values than faecal. Bates et al. (1985) have suggested that DNA values give a better indication of the bacterial content of rumen digesta than RNA values. This is because the RNA content varies with the microbial growth rate, diet and the time after feeding at which the samples are collected. Ling and Buttery (1978), however, found that the DNA determinations of their samples were more variable than the RNA values, and preferred to use RNA values. Ushida et al. (1985) have shown that losses of up to 10% of RNA may occur in bacterial samples that are frozen and stored at -20°C for 14d. Siddons et al. (1982) and McAllan and Smith (1983) have also shown that there may be losses of RNA upon freezing and thawing. This may have occurred in the present study, also, although all samples were frozen and only the diet and some of the ileal digesta samples were allowed to thaw once frozen, so this cannot explain why the ileal values were higher than the faecal values in some of the cases. McAllan and Smith (1969) reported that the recovery of DNA added to digesta samples was slightly higher than that of RNA, being 96$\pm$3% and 93$\pm$3% respectively.

Another possible explanation for the results obtained for the RNA determinations may be related to the method of detection. Both the orcinol and diphenylamine reactions suffer from lack of specificity, and it is very important, therefore, to ensure that appropriate measures are taken to avoid contamination of the RNA and DNA fractions by any interfering substances, and to check for interference, especially when using plant and bacterial samples. These interfering substances include hexose, sucrose, pentosans,

polyuronides, heptoses and their phosphates, polysaccharides, glycogen, protein, phosphates, trichloroacetic acid (TCA), and DNA (Smillie and Krotkov, 1960; Hutchison and Munro, 1961; Munro and Fleck, 1966) which interfere with the orcinol reaction, and protein, bile salts, glycogen, sugars, amino acids, sialic acid and various other substances (Burton, 1956; Croft and Lubran, 1965) which interfere with the diphenylamine reaction, although often to a minor extent. Also protein, peptides and TCA absorb light at 260nm, and therefore interfere with this method of detection. Both methods used in the present study for the detection of RNA are open to possible interference, more so than appears to be the case for the detection of DNA and this may be the cause of the unreliable results obtained for the RNA determinations. Also, the ileal digesta samples may contain more substances that are likely to interfere with the orcinol determination or 260nm detection than faeces samples, possibly leading to overestimation of RNA in these samples.

An attempt to remove interfering substances before measuring the RNA content, was made by applying the fraction from the alkaline digestion step in the RNA fraction to an anion exchange column (Smillie and Krotkov, 1960). McAllan and Smith (1969) found that although most of the interfering substances from digesta sample RNA fractions were removed by this procedure, some did still remain, and this is possibly also the case in the present study. The RNA values determined in the present study were considerably higher than values determined by the same method reported by Fleming and Wasilewski (1984) for pigs. In addition, the two methods for measuring RNA disagreed, and there was high variability within the data and poor agreement between duplicate analyses. Consequently, this data was considered to be completely unreliable and will not be discussed further.

There was a difference between the levels of DNA present in ileal and faeces samples in humans, with the faeces samples having a higher DNA content than ileal digesta, as would be expected due to

the microbial colonisation in the large intestine, although the difference was not as great as might be expected based on literature reports of the levels of bacteria found in various sections of the digestive tract (refer Section 1.4.4). The opposite was observed for the pig, with the daily faecal output of DNA being considerably lower than that in the ileal digesta, even though the concentration of DNA-N was slighty greater in the faeces. The daily excretion of dry-matter in the pig was much higher at the ileum than in the faeces and so when the total daily output of DNA-N was calculated, the ileal value was greater than that in the faeces.

There was no significant difference between the pig and human ileal DNA contents, therefore the ileostomised pig appears to have undergone the same extent of colonisation after the ileostomy operation as had occurred in the human subjects used in the study. It appears, however, that the human faeces samples had a higher DNA content than the pig faeces samples, which also does not agree with what would be expected according to the literature.

The DNA-N values found in the present study are lower than literature values reported for ruminants, and Ling and Buttery (1978) reported values for DNA-N:total N (mg/g) of 30.9 ± 3.8, for rumen bacteria samples. It would be expected that the levels of bacteria found in the gut of monogastric species would be considerably lower than those in the ruminant digestive tract. Salter and Coates (1971) reported ileal and faecal DNA-N and RNA-N values (expressed as a percentage of total insoluble N) for chickens of 1.6, 0.3, 4.7 and 1.4 respectively. These values are lower than the values found in the present study, and it is interesting to note that the faecal levels thus reported were lower than the ileal values. Fleming and Wasilewski (1984) reported ileal and faecal DNA and RNA values for pigs fed a range of diets, and found that the amounts excreted at either site varied with different types and levels of dietary fibre eaten. These workers found ileal and faecal DNA values ranging from approximately 0.1-2.6 and 0.5-0.8mg/day

respectively. These values are also considerably less than those reported here. The former study also found that with some diets, the ileal excretion of DNA was higher than that in the faeces. It is possible that the levels of cellular DNA in the gut are high, and even if the contribution of this is constant throughout the tract, there may be more degradation of this material by the gut microflora in the large intestine. The bacteria present in the small intestine after an ileostomy would not have as long to act on the digesta as those present in the large intestine due to the relatively rapid transit of material through the upper gut. This would result in the DNA measured in the faeces being largely due to bacteria, while the DNA at the terminal ileum may be of bacterial and cellular origin. It appears, therefore, that although the values found in the present study are high for monogastric species, the trends found at the two sites may be a real effect.

In summary, there appears to be a greater daily excretion of DNA-N in the faeces of humans than in ileostomy output, however these values were similar in the pig. There were also similar outputs in the two species at the ileum.

SECTION 6.2. THE USE OF DIAMINOPIMELIC ACID TO INDICATE THE LEVELS OF BACTERIA PRESENT IN DIET, ILEAL DIGESTA AND FAECES SAMPLES.

a. INTRODUCTION.

Diaminopimelic acid is an amino acid that only occurs in the cell walls of bacteria, and is not found in animal or plant cells. Diaminopimelic acid is often used as a bacterial marker in studies involving ruminants (Czerkawski, 1974; Ling and Buttery, 1978; Whitelaw et al., 1984; Ushida et al., 1985), and has been used to a lesser extent in studies with monogastric species, such as the rat (Mason and Palmer, 1973). The main disadvantage from using diaminopimelic acid is that the levels present in bacteria vary from one species to another, and it is completely absent from some species (Ling and Buttery, 1978). The type of diet may also affect the types and levels of bacteria present and thus the diaminopimelic acid content (Dufva et al., 1982). The advantages from using this marker are that it can be measured easily and is stable upon storage at -20°C (McAllan and Smith, 1983). Diaminopimelic acid can be detected by the usual method of amino acid analysis, that is ion-exchange chromatography after acid hydrolysis (Czerkawski, 1974; Edols, 1985).

The aim of the present study was to measure the amounts of diaminopimelic acid in digesta samples collected from ileostomised pigs and human subjects, and in the faeces of these species, to determine the levels of bacteria present in the respective samples. A comparison was then made between the two types of samples collected from each species to indicate the extent of colonisation at the terminal ileum following an ileostomy. A further comparison was made between the two species.

b. <u>METHODS AND MATERIALS.</u>

REAGENTS.

All reagents used were of AnalaR quality.
Hydrochloric acid (HCl), BDH, England.
DL-Diaminopimelic acid (DAP), Sigma Chemical Co., U.S.A.

CHEMICAL ANALYSIS.

The diet, ileal digesta and faeces samples were hydrolysed in duplicate as described in Section 4.2. and diaminopimelic acid was determined by ion-exchange chromatography. Diaminopimelic acid was found to elute in the same position as valine, however, using the normal amino acid analysis system. Therefore this had to be modified to separate these two peaks. This was achieved by extending the time of the first buffer by ten minutes. The position that diaminopimelic acid is eluted at is illustrated in Fig. 2.1 (Chapter 2).

STATISTICAL ANALYSIS.

The data were tested for homogeneity of the variance around the means using Barlett's test (Snedecor and Cochran, 1982), and a model which included terms for species (human versus pig), type (ileal versus faecal), and species*type, was fitted to the data. Reductions in sums of squares were used to determine levels of significance.

c. RESULTS.

The amounts of diaminopimelic acid (DAP) in all samples were low compared to the levels of other amino acids present in the samples. The elution of the diaminopimelic acid peak was on top of a buffer change during the amino acid analysis, which made the measurement of the peak difficult, however the use of an integrator greatly improved the accuracy of the measurements. There was no detectable DAP in any of the diet samples. The difference (expressed as a proportion of the mean) between duplicate analyses was generally less than 5%.

The mean amounts of diaminopimelic acid in the samples are given in Table 6.3. When the data were expressed as mg DAP/g dry-matter in the samples, there was a significant difference between ileal and faecal samples for both humans and pigs but there was no significant effect of species. However, when the data were expressed as mg DAP/day, there was a significant species*type interaction. Therefore an analysis of variance was performed within each species to determine the effect of type, and within each type to determine the effect of species. There was a significant difference between the human ileal and faecal DAP levels ($P<0.001$), but no significant difference ($P>0.5$) between pig ileal and faecal values. Also, there was no significant difference ($P>0.5$) between human and pig faecal DAP levels, but there was a significant difference ($P<0.01$) between human and pig ileal levels.

Table 6.3. Mean diaminopimelic acid (DAP) concentration (mg/g dry-matter) in ileal digesta and faeces samples from adult humans (65kg) and growing pigs (25kg) consuming a meat/vegetable/cereal/dairy products-based diet, and the mean daily DAP output (mg/day).

	HUMAN		PIG		S.E	Level of significance		
	Ileal (n=5)	Faecal (n=6)	Ileal (n=6)	Faecal (n=6)		S	T	S*T
DAP (mg/g DM)	0.5	2.3	0.6	2.0	0.38	NS	***	NS
DAP (mg/day)	31.3	72.9	56.7	62.8	8.61	*	***	***

d. DISCUSSION.

The concentrations of DAP in the faeces were greater than in the ileal digesta. The daily amount excreted was also higher in the faeces than at the ileum for both species. The difference between the ileal and faecal output was greater for humans than the pig. The faecal output was more than twice the amount at the ileum for humans, however in the pig the two values were similar, and in fact not statistically significantly different. It appears that the pig small intestine has undergone more extensive microbial colonisation following an ileostomy than that of the humans. It must, however, be pointed out that the amount of DAP varies from one bacterial species to another, and so when comparing the levels of DAP in two separate parts of the gut and in two different species of animals, the results need to be interpreted with some caution.

Diaminopimelic acid has been determined in the ileal digesta collected from preruminant calves (Guilloteau et al., 1986), and values of approximately 7-11mg/g N in the digesta reported. The levels in ileal digesta found in the present study of 11-19mg/g N were similar. The faecal values determined in the present study (43-48mg/g N) are also similar to faecal levels reported for the preruminant calf of around 30mg/gN (Guilloteau et al., 1980). Ling and Buttery (1978) reported daily duodenal DAP passage in sheep of 160-870mg which is considerably higher than the values found for humans and pigs in the present study. Czerkawski (1974) reported DAP values for rumen contents of 1.10-1.36 mg/g dry-matter, and Coto et al. (1985) reported values, also from rumen contents, of 2.20 mg/g dry-matter, which again are higher than the values in the present study. The levels of DAP reported for rumen digesta and ruminant duodenal digesta, however, would be expected to be higher than values found in the digestive tract of monogastrics. It appears that the values found in the present study are reasonable. The levels were greater in the faeces than at the ileum which would be expected according to other workers (refer Section 1.4.4). The pig ileum

appears to be more extensively colonised than the human ileum, but the reverse has been found in the present study for faecal levels.

SECTION 6.3. THE DETERMINATION OF DIETARY FIBRE DIGESTIBILITY TO INDICATE THE EXTENT OF BACTERIAL ACTIVITY IN THE SMALL AND LARGE INTESTINES IN HUMANS AND PIGS.

a. INTRODUCTION.

Dietary fibre is resistant to digestion by the enzymes secreted in the digestive tract of humans and pigs. However the microorganisms present in the gut are able to produce enzymes which can break down various components of dietary fibre. Therefore, the extent of digestion of dietary fibre components may indicate the extent of bacterial activity in the gut, and the possible extent of protein digestion carried out by the gut bacteria. It should be recognised, however, that not all bacteria are able to break down both protein and carbohydrate, and some strains break down the two dietary components to different degrees (Drasar and Hill, 1974; Hungate, 1978). The aim of the present study was to determine the digestibility of the various dietary fibre components at the terminal ileum and in the faeces of humans and pigs. This would then allow a comparison to be made of the apparent levels of activity at these two sites for each species, and a comparison between the species.

There are several methods for the analysis of dietary fibre in foods (James and Theander, 1981). The Southgate method (1976) allows determination of the individual components of dietary fibre by separation of the fractions, due to solubility, through a series of extractions. The fractions are hydrolysed to give the component sugars and these are measured by colorimetric detection methods, gas-liquid chromatography (GLC) or high performance-liquid chromatography. Other methods measure the soluble and insoluble fibre components, or neutral and acid-detergent fibre components (based on the extraction method), and these have been reviewed by Southgate et al. (1978). These methods, however, do not give estimates of the digestibility of the individual components, or the

pectin, hemicellulose, cellulose and lignin fractions of dietary fibre. The method used in the present study was based on the Southgate method.

b. METHODS AND MATERIALS.

REAGENTS.

Ethanol, Acetone, Sulphuric acid (H_2SO_4), Sodium hydroxide (NaOH), Octan-2-ol, Acetic anhydride, Sodium borohydride, Xylose, Arabinose, BDH, England.

Phosphate buffer: 10g Sodium dihydrogen phosphate (NaH_2PO_4, Reidel-De Haen Ag Seelze-Hannover.) and 1.5g Disodium hydrogen phosphate (Na_2HPO_4, May and Baker Ltd., Australia) dissolved in 500ml distilled water, pH 6.0.

Termamyl (Novo, 120KNU/g), May and Baker Ltd., Australia.

Protease (Streptomyces griseus type XIV), Sigma Chemical Co., U.S.A.

Amyloglucosidase, E.C. 3.2.1.3., Boehringer-Mannheim N.Z. Ltd., N.Z.

Diethyl ether, Boric acid, May and Baker Ltd., Australia.

orthophosphoric acid, Ajax Chemicals, Australia.

Dinitrosalicylic acid (DNS) solution: Heat 500ml water to 30°C, add 10g DNS (BDH Chemicals Ltd., England) and dissolve at room temperature. Add 300g sodium potassium tartrate (May and Baker Ltd., Australia). Cool and make up to 1l.

Sodium chloride (NaCl).

3,5-dimethylphenol, Fucose, Koch Light Lab., England.

N-methylimidizole, Deoxyglucose, Mannose, Sigma Chemical Co., U.S.A.

Ammonium hydroxide (NH_4OH), J.T. Baker Chemical Co., U.S.A.

Glacial Acetic acid, Ajax Chemicals, Australia.

Potassium hydroxide (KOH), May and Baker Ltd., Australia.

D-glucuronic acid, Sigma Chemical Co., U.S.A.

Rhamnose, Hopkins and Williams Ltd., England.

Glucose, Glaxo, N.Z.

Galactose, Difco Lab., U.S.A.

CHEMICAL ANALYSIS.

1. PRELIMINARY EXTRACTION USING HOT ORGANIC SOLVENTS. Duplicate 5g samples of the freeze-dried and finely ground diet, ileal digesta and faeces samples collected as described in Section 4.2, were accurately weighed into preweighed 100ml beakers and 25ml of 85% ethanol were added. The contents of the beaker were stirred while being brought to the boil on a hot plate. The solvent was decanted off, and filtered through Whatman 541 paper. The extraction was repeated twice more with 85% ethanol, then with three portions of hot acetone. The residue was allowed to air-dry, and then carefully scraped back into a tared beaker, and the beaker reweighed. The residue was finely ground and stored in an air-tight container for subsequent analysis. For the analysis of the diets used in Chapter 4, the ethanol extracts were made up to 100ml and the soluble sugar content determined after hydrolysis of the extract in 2M H_2SO_4, by the Dinitrosalicylic acid (DNS) method (Miller, 1959).

2. STARCH DIGESTION. Duplicate 200-300mg samples of the residue obtained after the above extraction procedure were accurately weighed into 50ml centrifuge tubes, and 4ml of boiling deionised water was added, and mixed. The tubes were placed in a boiling water bath for an hour (Schweizer and Wursch, 1981), with frequent stirring (Southgate and White, 1981) to gelatinise the starch. After the 1h incubation, 4ml of phosphate buffer and 25µl of Termamyl solution were added and mixed, then the tubes were covered and placed in a sand bath at 95°C for 1h with frequent shaking. The tubes were cooled and adjusted to pH7.5 by adding 0.8ml of 0.57M NaOH. Then 0.1ml of a 1.0mg/100µl solution of protease were added and mixed. The tubes were covered and placed in a water bath at 37°C for 1h with frequent shaking (Rasper, 1981; Prosky _et al._, 1984). The tubes were then cooled and adjusted to pH4.6 by adding 0.25ml 3.29M phosphoric acid (Southgate and White, 1981; Prosky _et al._, 1984). An aliquot, 0.2ml, of a 10mg/ml solution of amyloglucosidase was added and mixed (Asp _et al._, 1983) The tubes were covered and

incubated overnight at 37°C, with stirring in the initial stages. The tubes were cooled then centrifuged at 2500rpm for 10mins. The supernatant was decanted off and collected in a 25ml volumetric flask. The residue was washed with 2 portions of 5ml of warm water, and the washings added to the flask. The flask was made up to volume and stored at -20°C for subsequent analysis. The glucose from starch in the diet (refer Table 4.4) was measured on this fraction by the DNS acid method.

3. PECTIN FRACTION. To 5ml of the supernatant collected in step 2 were added 4 volumes of absolute ethanol, and mixed. The mixture was allowed to stand for 10mins to allow the precipitate to form, then centrifuged for 10mins at 2500rpm, the supernatant discarded, and the precipitate washed and recentrifuged. One ml of 1M H_2SO_4 was added to the residue and mixed, and hydrolysis carried out at 120°C for 1h (Laine et al., 1981). The hydrolysates were then made up to a volume of 5ml, and stored for subsequent analysis of uronic acids by the colorimetric method and sugars by GLC.

4. HEMICELLULOSE FRACTION. The residue from step 2 was resuspended in 10ml of 1M H_2SO_4, and the tubes covered and heated in a boiling water bath for 2.5h, with mixing after 1h. The tubes were then cooled, and an equal volume of ethanol was added and mixed. The tubes were centrifuged and the supernatant collected in a 50ml volumetric flask. The residue was washed in 50% ethanol and recentrifuged, with the washings added to the flask. The residue was then washed with ethanol followed by diethyl ether, with the washings discarded, and the residue air-dried. The supernatants were made up to volume and stored at -20°C for subsequent analysis. The uronic acids were measured by the colorimetric method, and the other sugars by GLC.

5.CELLULOSE FRACTION. The residue from step 4 was chilled and had 10ml of chilled 72% wt/wt H_2SO_4 added and mixed carefully. The tubes were left at 0-4°C for 48h with stirring in the initial stages. The

contents of the tubes were filtered through preweighed sintered glass funnels and washed thoroughly with water, with the filtrate collected in a 50ml volumetric flask. The filtrates were made up to volume and stored at -20°C for subsequent analysis. The glucose from cellulose was measured by the DNS method.

6.LIGNIN FRACTION. The residue collected on the sintered glass filters was washed with ethanol followed by diethyl ether, and the residue was dried by heating in an oven (80°C), allowed to cool, and then reweighed.

7.DETERMINATION OF REDUCING SUGARS BY THE DNS METHOD. One ml of the sample to be measured was mixed with 2ml of DNS solution, then the tube placed in a boiling water bath for 15min. The cooled samples then had 3ml of water added and after mixing, the absorbance was measured at 580nm (Miller, 1959). A series of dilutions of standard glucose was treated in the same way and used to prepare a standard curve.

8.DETERMINATION OF URONIC ACIDS. To 0.3ml of the hydrolysate was added 0.3ml of a solution containing 2g of NaCl and 3g of boric acid/100ml, and mixed. Then 5ml of concentrated H_2SO_4 were added and mixed carefully. The tubes were placed in a heating block at 70°C for 40min. After cooling, 0.2ml of dimethylphenol solution was added and mixed, and the absorbance read at 400nm and 450nm 10-15min later. A series of glucuronic acid standards were treated in the same way to obtain a standard curve. The 400nm readings were subtracted from the 450nm readings (Englyst and Hudson, 1987).

9.DETERMINATION OF SUGARS AS THEIR ALDITOL ACETATES BY GAS-LIQUID CHROMATOGRAPHY. The determination of monosaccharides by GLC requires that they are derivatised first. To 1ml of each hydrolysate, 0.25ml of 10M ammonium hydroxide (NH_4OH) and 5µl of octan-2-ol were added, and the solutions tested for alkalinity. Then 0.1ml of a freshly prepared solution of 100mg of sodium borohydride/ml of 3M NH_4OH was

added. After mixing, the tubes were left at 40°C for 1h, then 0.15ml of glacial acetic acid were added and mixed. To 0.5ml of the acidified solution were added 0.5ml of N-methylimidizole and 5ml of acetic anhydride and mixed. This was left at room temperature for 10min, then 0.6ml of ethanol were added and mixed. After 5min, 5ml of water were added and the tubes were placed in a water bath at room temperature, then 5ml of 7.5M KOH were added followed by another 5ml a few minutes later. The contents of the tubes were mixed by inversion and left to separate into 2 layers. The top layer was carefully removed for GLC, and stored at 5°C for susequent analysis (Englyst and Cummings, 1984). Standard monosaccharides were also derivatised. Deoxy-glucose was used in all samples as an internal standard.

One to five µl of each alditol acetate were analysed using a Gas Chromatograph (1440-10 Varian Aerograph), and the conditions used are shown in Fig. 6.1, which shows a typical chromatograph of the individual sugar peaks. The peak areas for each sugar were measured and the amount calculated by reference to the internal standard and to the peak areas of standard amounts of each sugar (Sawardeker et al., 1965).

STATISTICAL ANALYSIS.

The data for the various fibre components and constituent sugars and uronic acids were determined using equation 1.1 (refer Section 1.3.1). The data were tested for homogeneity of variance around the means using Bartlett's test (Snedecor and Cochran, 1982), and a model which included terms for species (human versus pig), type (ileal versus faecal), and species*type, was fitted to the data. Reductions in sums of squares were used to determine levels of significance.

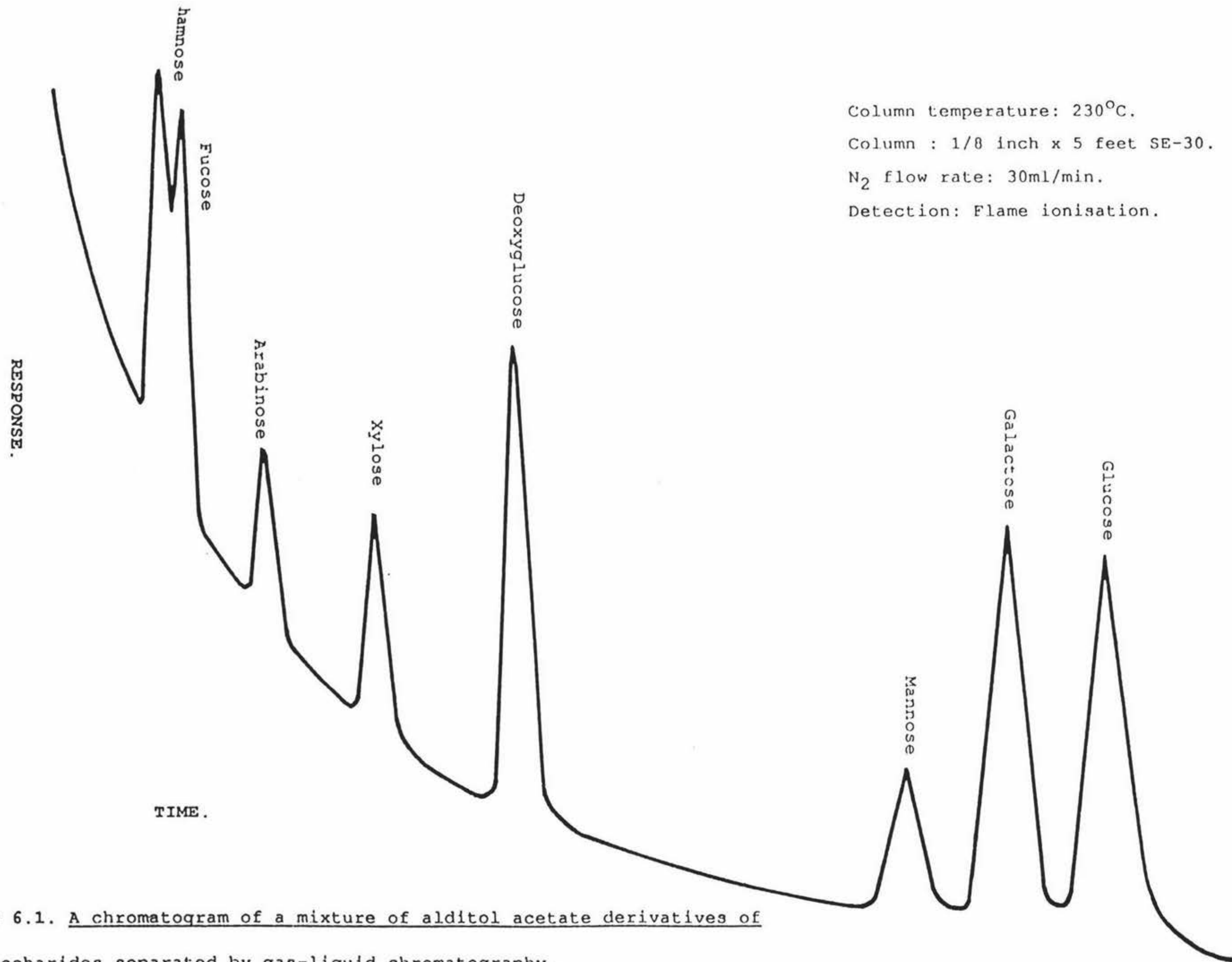


Figure 6.1. A chromatogram of a mixture of alditol acetate derivatives of monosaccharides separated by gas-liquid chromatography.

c. RESULTS.

The sugar composition of the fibre fractions in the diet used in the present study is given in Table 6.4. There were only traces of lignin found in all of the samples analysed in the present study. The agreement between duplicate analyses was often poor (although generally less than 20% difference expressed as a proportion of the mean), and the variability within samples large, with the ileal values being more variable than the faecal values. The mean results for the digestibility of the fibre components are given in Table 6.5. There was a statistically significant interaction for all of the components measured, therefore an analysis of variance was performed within type for each species, and within species for each type, and the results are shown in Table 6.6. There were significant differences between the ileal and faecal measurements for all components for both species. There were no significant differences between the two species for the ileal digestibility of hemicellulose and the faecal digestibility of pectin, but significant differences were found for the digestibilities of the other components for both type and species.

The faecal digestibilities of all components measured were higher than the comparable ileal digestibilities for both humans and pigs. The digestibility of most components, except for cellulose, was almost complete when measured in the faeces for both species.

Table 6.4. The sugar composition (mg/g dry-matter) of the dietary fibre fractions extracted from a meat/vegetable/cereal/dairy product based diet.

Component	Composition*1
PECTIN FRACTION*2	
Rhamnose	0.23
Arabinose	1.10
Xylose	1.81
Mannose	0.51
Galactose	1.26
Glucose	4.37
Uronic acids	0.04
Total pectin	7.51
HEMICELLULOSE FRACTION	
Rhamnose	4.30
Arabinose	31.52
Fucose	trace
Xylose	30.21
Mannose	9.60
Galactose	22.19
Glucose	15.56
Uronic acids	0.13
Total	113.51
CELLULOSE	3.80
LIGNIN	trace
STARCH	255.65

*1 Based on chemical analysis.

*2 There is incomplete separation of each fibre component; water-soluble hemicelluloses occur in the pectin fraction.

Table 6.5. Mean apparent ileal and faecal digestibilities (%) of fibre components*1, for adult humans (65kg) and growing pigs (25kg) fed a meat/vegetable/cereal/dairy products-based diet.

Component fraction	HUMAN Ileal	HUMAN Faecal	PIG Ileal	PIG Faecal	S.E.	Level of significance S	T	S*T
	(n=5)	(n=6)	(n=6)	(n=6)		S	T	S*T
Pectin	86.9	95.1	92.6	98.7	2.31	***	***	***
Hemicellulose	44.7	96.1	53.0	99.1	11.49	**	***	*
Cellulose	8.1	53.5	47.6	71.2	1.44	***	***	***

*1 Digestibility values for the individual sugars and uronic acids are given in Appendix 4, Table 4.1.

Table 6.6. Levels of significance for the differences in fibre component digestibilities between type (ileal and faecal) within species (human and pig) and between the species within type.

Fibre fraction	TYPE		SPECIES	
	Pig	Human	Ileal	Faecal
Pectin	***	**	**	NS
Hemicellulose	***	***	NS	***
Cellulose	***	***	***	**

d. DISCUSSION.

The determined level of dietary fibre (34g/day for diet A) was similar to the amount calculated by reference to food tables (30g/day), (Paul and Southgate, 1978), which are mostly derived by a similar procedure. This agreement between determined and tabulated values gives confidence regards the chemical method used in the present study for the determination of fibre. There may have been some contamination of the fibre fractions determined in the present study due to sugars from bacteria and from endogenous glycoprotein of mucus.

The digestibility of cellulose determined in the present study (8%) was low when measured in human ileal digesta. Other workers have reported cellulose digestibility values at the terminal ileum of 15.5% (Holloway et al., 1978) and upto 26% (Sandberg et al., 1981), which are higher than that found in the present study. The digestibility of hemicellulose before the terminal ileum in humans was 44.7%, with the individual sugar digestibilities ranging from 26.8-77.4% for galactose and mannose respectively. These values are slightly lower than the digestibility of the hemicellulose sugars reported for human ileostomy patients of 72.5% (Holloway et al., 1978) and 65-83% (Holloway et al., 1980). Sandberg et al. (1981) calculated hemicellulose levels on the basis of arabinose and xylose recoveries, and reported 0-31% digestibilities, which are lower than the values for arabinose (38.9%) and xylose (54.1%) found in the present study. The values from the present study appear to be somewhere in the middle of these other reported values, and so it appears that some digestion of hemicellulose does occur in the small intestine. The digestibility of pectin was almost complete in the human ileostomate in the present study. Holloway et al. (1983) reported digestibilities of citrus pectin of only 15.3-46.6%, and Sandberg et al. (1981) reported digestibilities of 0-30%, which are lower than the values found in the present study.

The digestibility of cellulose (53.5%) in the human faeces samples is also within the range of values reported by other workers of 78% (Holloway et al., 1978) and 6-40% (Cumming and Englyst, 1987). The human faecal hemicellulose sugar digestibility values in the present study ranged from 81.4-98.5%, for rhamnose and xylose respectively, with the total hemicellulose digestibility being 96.1%. Holloway et al. (1978; 1980) reported similar faecal hemicellulose digestibility values of 95-97%. Slavin et al. (1983) reported apparent faecal digestibilities of hemicellulose monosaccharides of 76-89% for xylose and arabinose respectively, for a low fibre diet, and 43-73% for mannose and arabinose respectively, for a high fibre diet. In the present study, the digestibility of pectin components in the human faecal samples ranged from 94.9-99.8% for uronic acids and rhamnose respectively. Similar values have been reported by Cummings and Englyst (1987), and by Holloway et al. (1983). It appears, therefore, that in humans the digestion of all fibre components except cellulose is almost complete by the end of the large intestine.

In the pig, the digestibility of cellulose (47.6%) before the ileum was higher than values reported by other workers of 0-25% (Cranwell, 1968; Kass et al., 1980; Millard and Chesson, 1984). The value for the ileal digestibility of pectin was also slightly higher than the values given by Millard and Chesson (1984) for the pig. In contrast, the ileal digestibility values for hemicellulose (53.0%) found in the present study were similar to values for the pig reported by other workers of up to 47% (Keys and DeBarthe, 1974; Kass et al., 1980; Millard and Chesson, 1984).

The faecal digestibility of cellulose in the present study (71.2%) in the pig is also slightly higher than values reported by other workers (Kass et al., 1984), however the values appear to be quite varied and Stanogias and Pearce (1985) reported faecal cellulose digestibilities of 13-93% depending on the source of the fibre in the diet. Cranwell (1968) also suggested that the extent of fibre

digestion in the large intestine depended on the source of fibre consumed. The digestion of pectin and hemicellulose was virtually complete in the faeces of pigs in the present study, which has also been found by other workers (Stanogias and Pearce, 1985). The hemicellulose values have also been found to vary according to the origin of the fibre fed (Stanogias and Pearce, 1985), and Kass et al. (1984) reported slightly lower faecal hemicellulose digestibilities than found in the present study for the pig.

A comparison of the extent of the digestion of fibre components in pigs and humans both at the ileum and in faeces, has shown that while there were statistically significant differences between the species for the ileal digestibility of pectin and the faecal digestibility of hemicellulose, all the digestibility values for these two fibre fractions were quite similar in absolute terms. The digestion of pectin was almost complete at the terminal ileum in both species, while the digestibilities of the other components were considerably lower at this site. The digestibility of cellulose was considerably higher in the pig than in humans at both sites. It must be pointed out, however, that the intestinal microbial populations of the two species may be different, and while the digestion of fibre components generally appeared to be similar, this may not mean that the microbial digestion of protein was also similar between the two species.

The extent of digestion of fibre components at the ileum and in the faeces of humans and pigs generally appears to be similar to the findings reported by other workers, although the digestibility of some components found in the present study were considerably higher. This may have been due to the diet used, which was quite digestible, as well as being reasonably processed and refined, and this may also affect the ease with which the fibre components can be utilised by gut microorganisms.

It appears, therefore, that some microbial activity has occurred, which is more substantial in the large intestine than the small

intestine. It also appears to be greater at the ileum in the pig than humans, which may be due to greater colonisation of the pig small intestine.

The extent of fibre digestion is only an indication of the microbial activity, and fermentation of carbohydrate is more extensive than for protein and fat (Cranwell, 1968). Some microorganisms present in the small and/or large intestines may also be able to ferment carbohydrate, but may be more limited in their ability to ferment protein (Drasar and Hill, 1974; Hungate, 1978). The extent of microbial protein digestion, therefore, may not be as great as indicated by the fermentation of some of the dietary fibre components measured in the present study.

6.4. SUMMARY AND CONCLUSION.

The results of the present study suggest that a considerable microflora exists at the terminal ileum of both pigs and humans, following an ileostomy. The daily excretions of DAP and DNA-N were higher in faeces than ileostomy output in the human subjects, and the faecal digestibility coefficients of dietary fibre were also higher than the ileal values. In the pig, however, the findings were less conclusive, with the daily output of DNA-N being greater at the terminal ileum than in the faeces, while the DAP excretion was similar at the two sites. In contrast, the faecal digestibilities of the fibre components were higher than the ileal values for the pig. The DNA values for the pig ileal digesta and faeces appear to be anomalous, although it is possible that there may be more interfering substances in the pig ileostomy output than in the humans, such as endogenous protein, bile and mucus components. Other workers have also reported higher DNA levels at the ileum of monogastric species (Salter and Coates, 1971; Fleming and Wasilewski, 1984), and the DNA values reported in the present study may not be a real indication of the levels of bacteria present in the gut. Some DNA, however, may be of cellular origin, and this may be greater in the small intestine, because the bacteria have less time to degrade the cellular DNA due to rapid transit, whereas the faecal levels may reflect the residual bacterial DNA. In light of the potential methodological problems involved in the determination of these bacterial markers, the results from the present study need to be interpreted with some caution.

In general, it appears that the human terminal ileum is less colonised after an ileostomy than the large intestine. It is also likely that the extent of microbial colonisation in the ileostomised animal is greater than would normally exist in the terminal ileum (Gorbach <u>et al.</u>, 1967), and therefore it is not expected that the ileostomy subject would be a perfect model for the intact subject. However, studies measuring volatile fatty acid production in

terminal ileal contents (Macfarlane et al., 1986) or breath hydrogen in ileostomates (Chapman et al., 1985; Englyst and Cummings, 1986) suggest that the levels of bacterial fermentation are low at this site in both intact and ileostomy subjects.

The pig terminal ileum appears to have undergone more extensive colonisation following an ileostomy than in human subjects. This may be because the pig has already had some degree of colonisation of the small intestine before the formation of the ileostomy, which then becomes elevated post-surgery. There was no significant difference between humans and pigs for the ileal excretion of DNA-N, but a significantly higher ileal DAP output in the pig. Also, the digestibility coefficients of fibre fractions were generally higher at the terminal ileum of pigs than humans. This suggests that the ileostomised pig has some limitations as a model for ileostomised humans, and possibly alternative methods for the collection of ileal digesta from pigs would lead to improvements.

The results from the present study tend to show that the terminal ileum is highly colonised following an ileostomy. It is not known, however, to what extent this would affect the determination of protein digestibility at this site. While the activities of the bacteria in the small intestine appear to be reasonably extensive, as indicated by the digestion of fibre, this may not mean that the same extent of protein digestion had occurred. Other studies (Chapman et al., 1985; Englyst and Cummings, 1986; Macfarlane et al., 1986) have provided contradictory evidence to suggest that the extent of bacterial fermentation is low in the ileum of ileostomates, and it is therefore difficult to draw any strong conclusions as to the degree of suitability of the ileostomy subject for the determination of ileal protein digestibility coefficients in humans.

OVERALL SUMMARY AND CONCLUSION.

The main aim of the present study was to investigate if a difference exists between the ileal and faecal assays, for the determination of amino acid digestibility in humans, using the ileostomy subject for the collection of ileal digesta. The ileal assay is the preferred method in work with other monogastric species. The present study has shown that a difference between the two methods does exist for the digestibility of most amino acids from a mixed diet. The diet used was based on meat, vegetables, cereals and dairy products, and was reasonably low in dietary fibre and fairly processed, therefore it is likely that for less refined foods an even greater difference would exist. Future work should investigate the digestibilities of a range of food ingredients and diets, to determine if the ileal assay is the most accurate method for a variety of foods.

It is important to have a routine method to measure the protein digestibilities of human foods and diets, and a model animal would be useful for this. The present study demonstrated close agreement between pigs and humans for the apparent ileal digestibility of amino acids for a mixed diet.

To allow a more precise comparison to be made between the two species for the ileal assay, the present work included a measure of the endogenous amino acid contribution at the terminal ileum of both species. This was carried out by collecting ileal digesta following the consumption of a protein-free meal, and using Celite as a non-digestible marker compound, which had been shown to be suitable for use in human studies. The total flows of endogenous amino acids were different for the two species, although the amino acid compositions were similar. The estimates of endogenous amino acid flow were used to correct the apparent amino acid digestibilities in each species to give true coefficients. There was closer agreement between the two species when the true ileal digestibilities were compared than

with the apparent values. It appears that the pig is a suitable model animal for the determination of ileal amino acid digestibilities at least for a mixed diet. Future studies should compare the species for a range of ingredients, especially with varying levels of dietary fibre.

A potential criticism of the present approach is the use of the ileostomised subject for the collection of ileal digesta, as the terminal ileum is known to host a greater microbial population in these subjects than is found in the intact small intestine. An attempt was made in this study to estimate the levels of bacteria in the ileostomy output and in the faeces of the pig and humans. For deoxyribonucleic acid (DNA) and diaminopimelic acid (DAP), it was found that the faecal levels were higher than the ileal levels in humans, but in the pig the opposite was observed for DNA, and the DAP levels were similar at the two sites. It was concluded that the ileum appears to have undergone considerable colonisation following an ileostomy, which was almost as extensive as that in the large intestine, and that the levels of bacteria appear to be greater at the ileum for the pig than humans. The rapid transit of digesta through the small intestine, however, may mean that the bacteria do not have enough time to have a significant effect on the overall digestion. To investigate this possibility, the extent of fibre digestion was also determined in the present study. The break-down of most fibre components was considerably less at the terminal ileum than in the faeces, although the extent of fibre digestion appeared to be greater in the pig than in the humans. It must be noted, however, that the bacterial fermentation of dietary fibre does not necessarily indicate that the same extent of protein digestion had occurred. Overall, it appears that the ileostomised subject is not a perfect model for the intact human. Future work should determine the digestibility of amino acids in human foods and diets using the pig as a model animal but using alternative means of collecting ileal digesta.

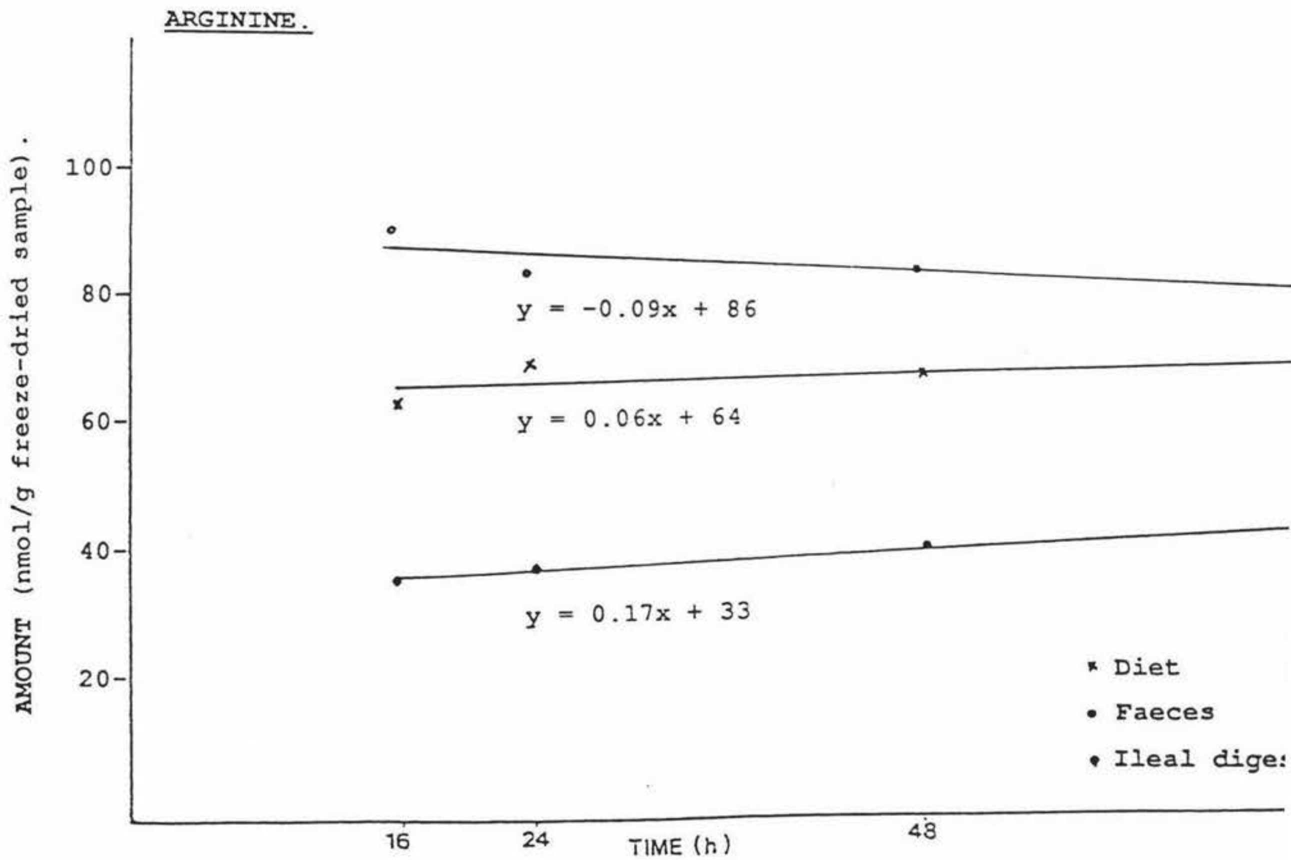
The present work has also demonstrated that the pig is a suitable model for humans for the digestibility of dry-matter, the faecal digestibility of gross energy and for the digestibility of dietary fibre. If it is more convenient, although less accurate, to use the faecal method for determining amino acid digestibility, the pig appears to be a suitable model for humans for this purpose also.

In conclusion, the present study provides evidence to show that a difference does exist between the ileal and faecal assays for determining the digestibility of dietary amino acids in humans, and that the pig may be considered useful as a model animal for humans in the determination of the digestibility of dietary protein.

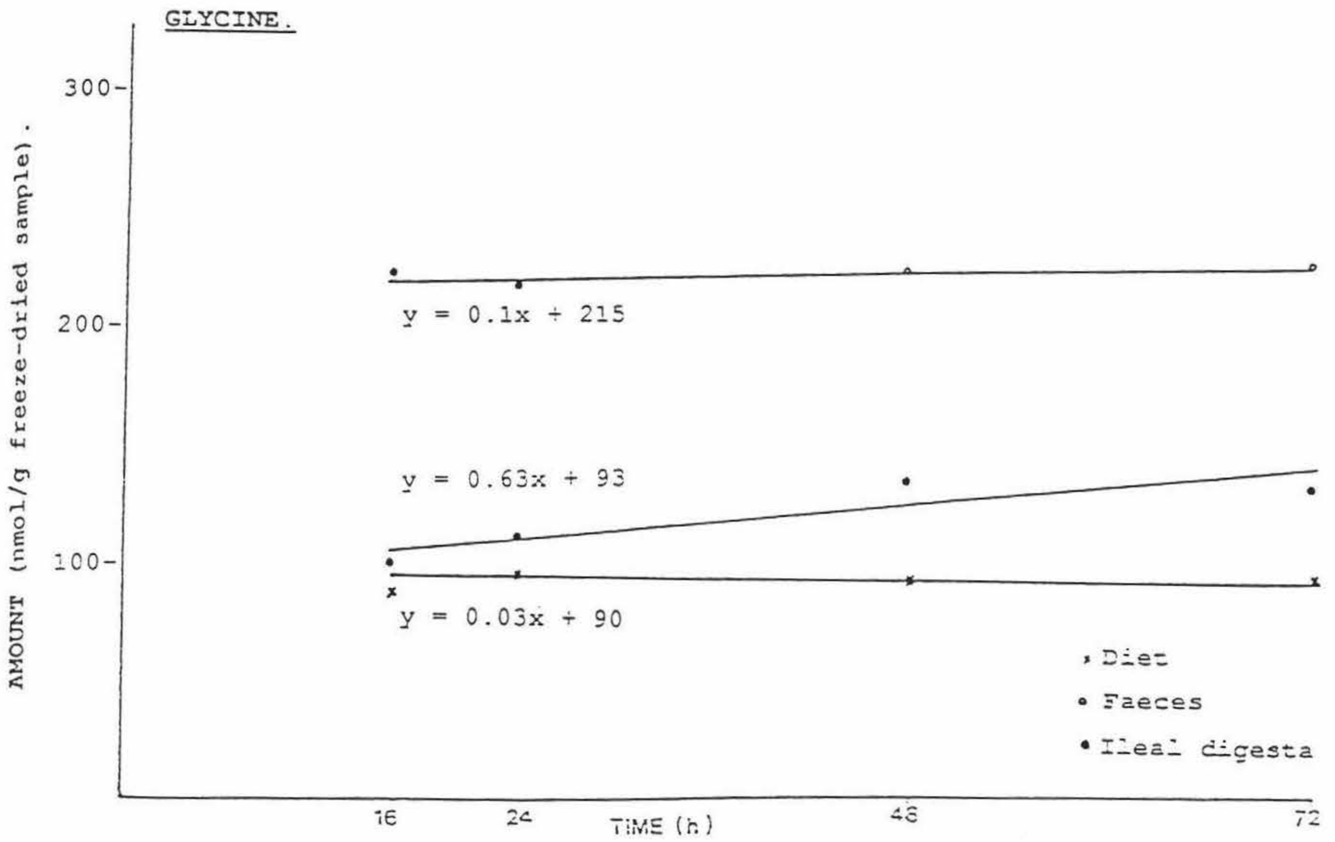
APPENDIX 1.

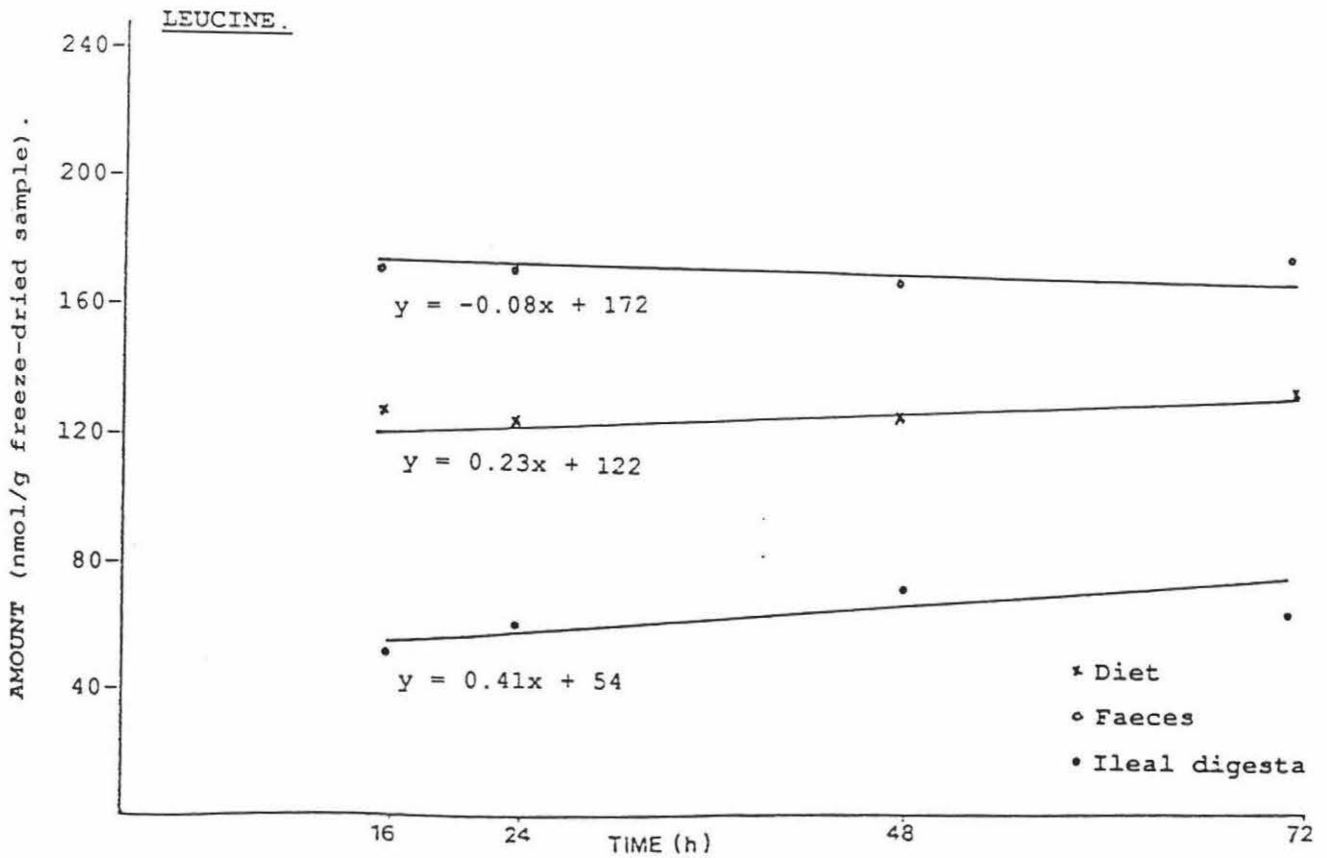
The effect of hydrolysis time during amino acid analysis on the mean[*1] yield of amino acids from diet, ileal digesta and faeces samples (refer Section 2.1, part 2).

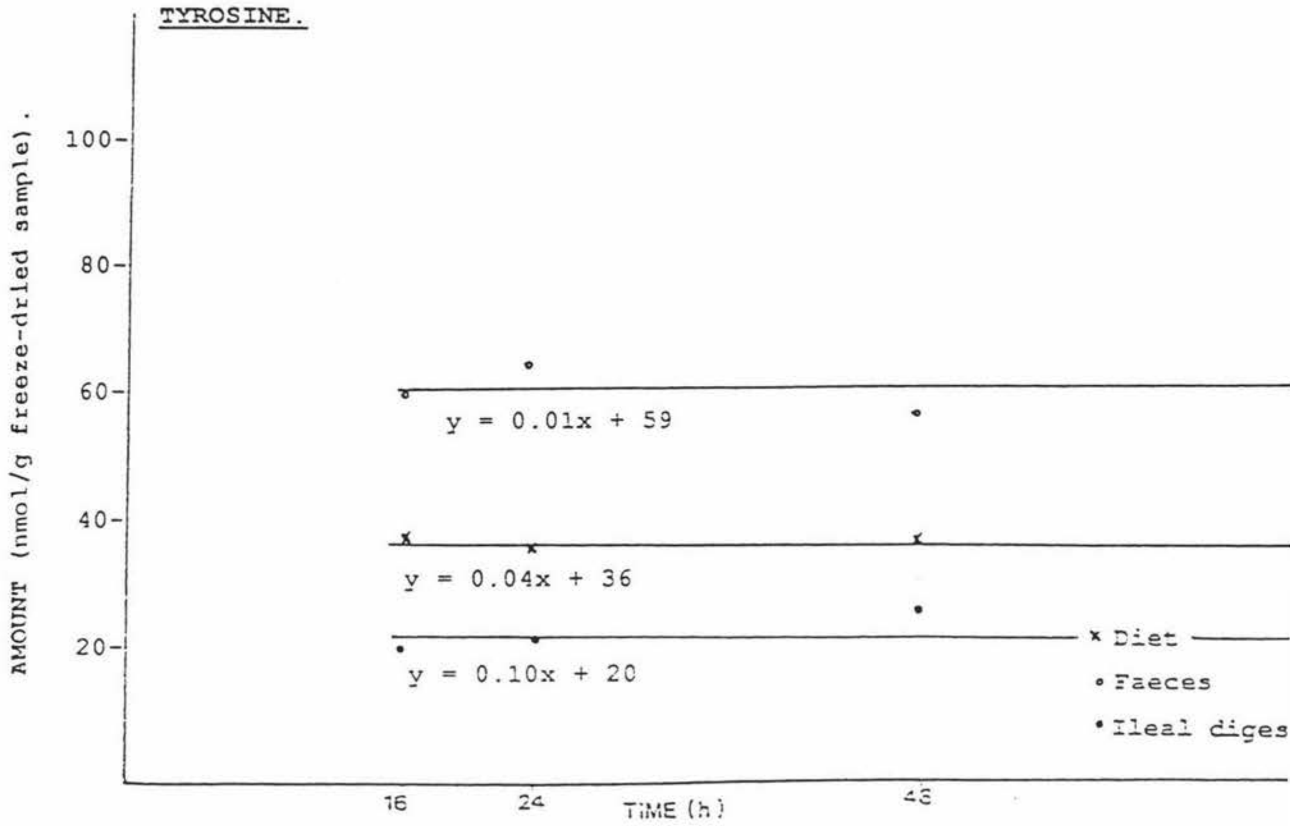
Linear response.



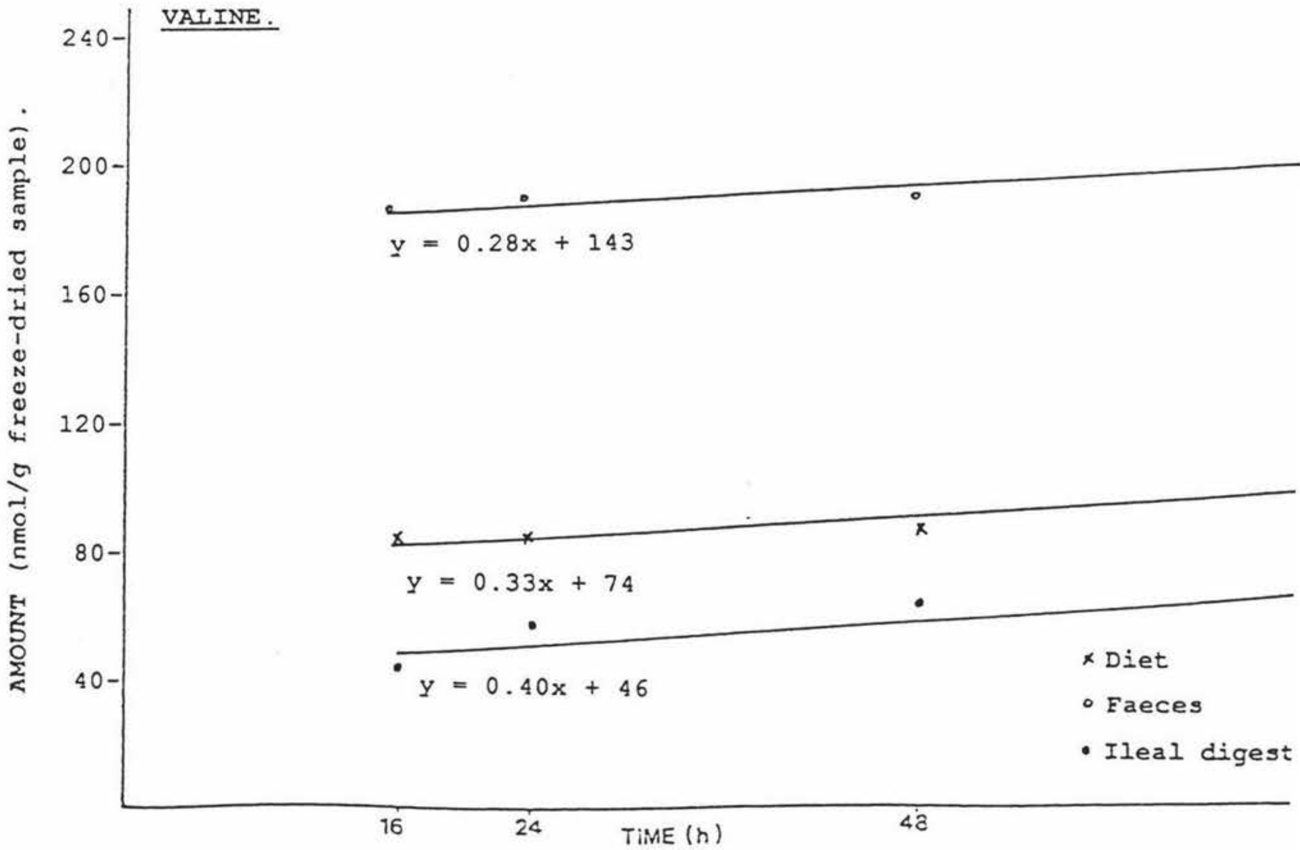
*1 In this and subsequent graphs the points are the means of duplicate analyses.


GLYCINE.
AMOUNT (nmol/g freeze-dried sample).
300-
200-
100-
y = 0.1x + 215
y = 0.63x + 93
y = 0.03x + 90
Diet
Faeces
Ileal digesta
16
24
48
72
TIME (h)


LEUCINE.
AMOUNT (nmol/g freeze-dried sample).
240-
200-
160-
120-
80-
40-
y = -0.08x + 172
y = 0.23x + 122
y = 0.41x + 54
Diet
Faeces
Ileal digesta
16
24
48
72
TIME (h)

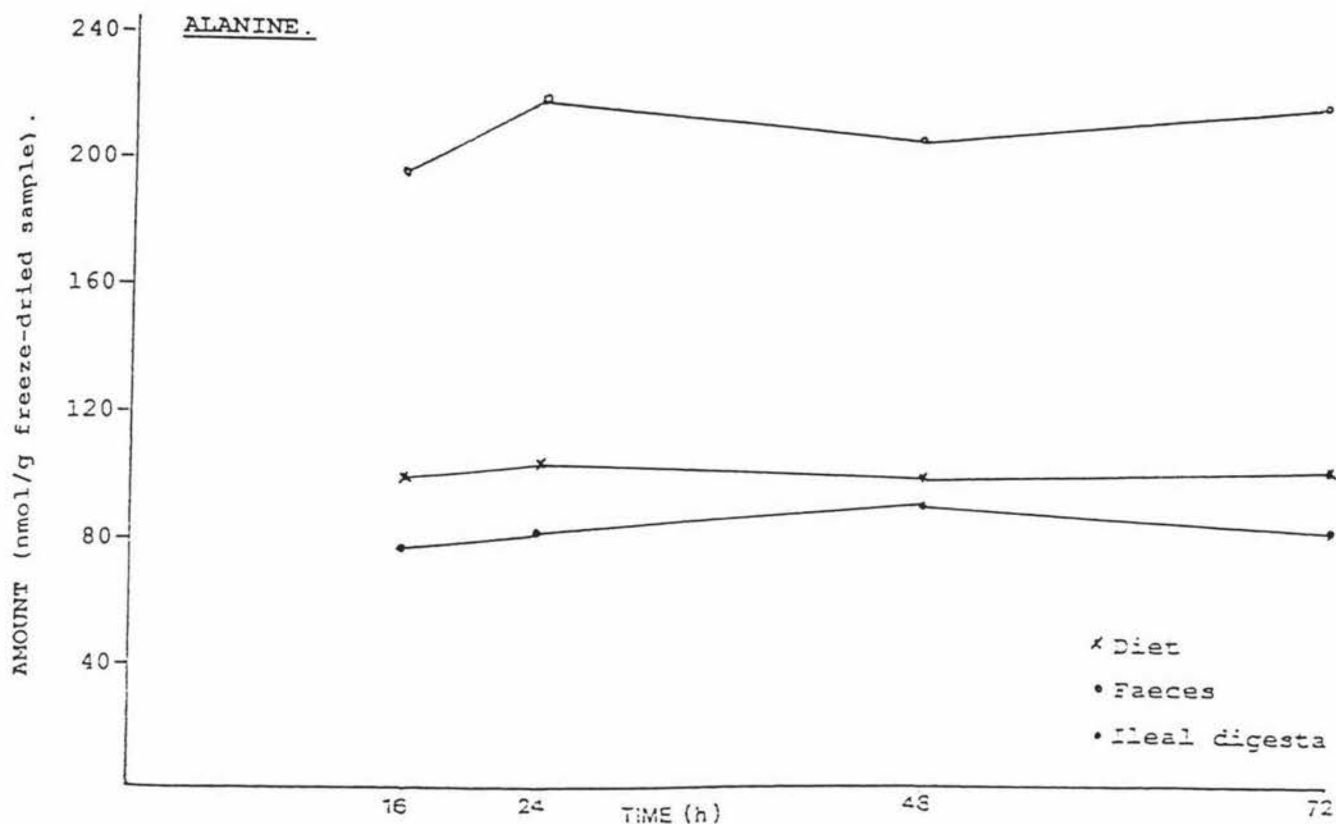


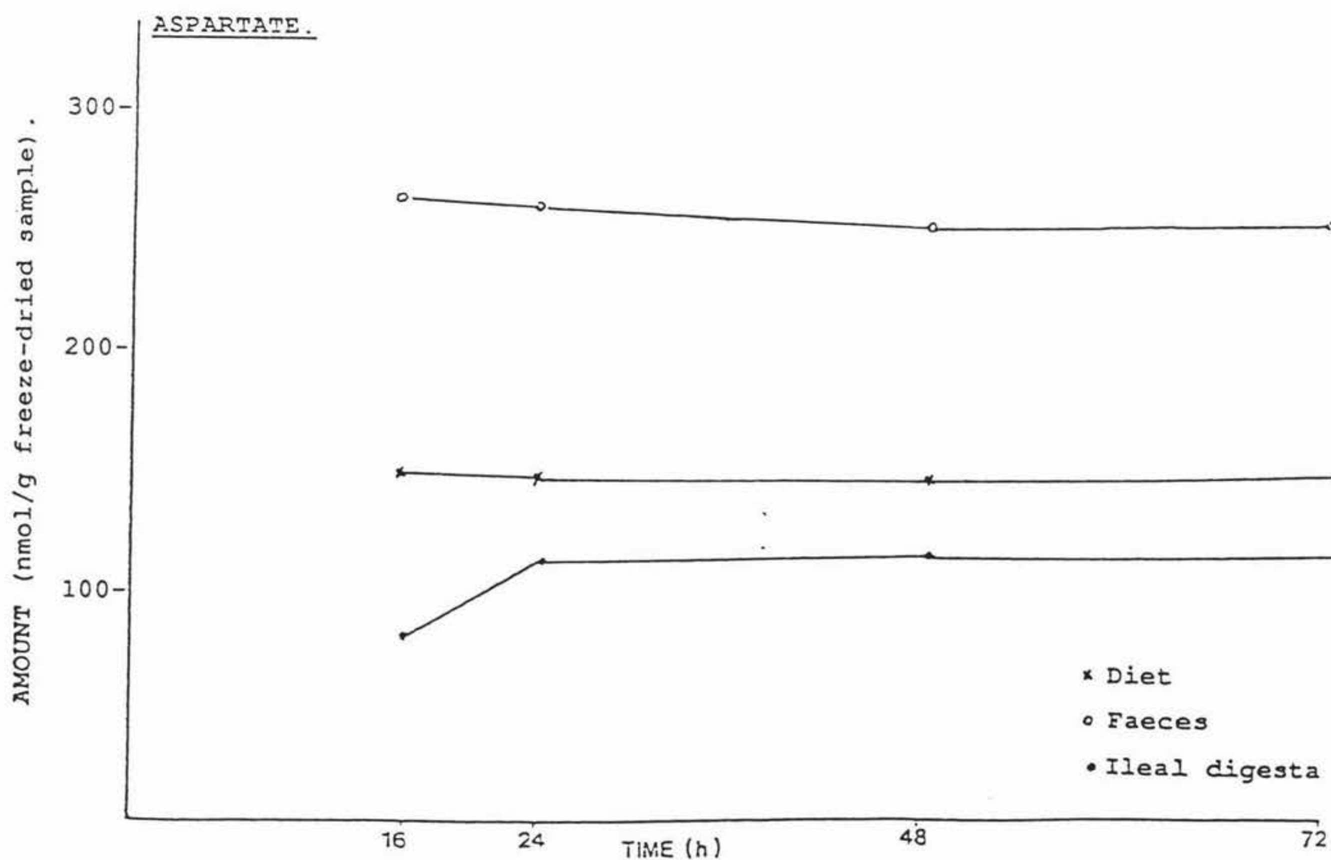
TYROSINE.
AMOUNT (nmol/g freeze-dried sample).
100-
80-
60-
40-
20-
y = 0.01x + 59
y = 0.04x + 36
y = 0.10x + 20
× Diet
∘ Faeces
• Ileal diges
16
24
TIME (h)
48

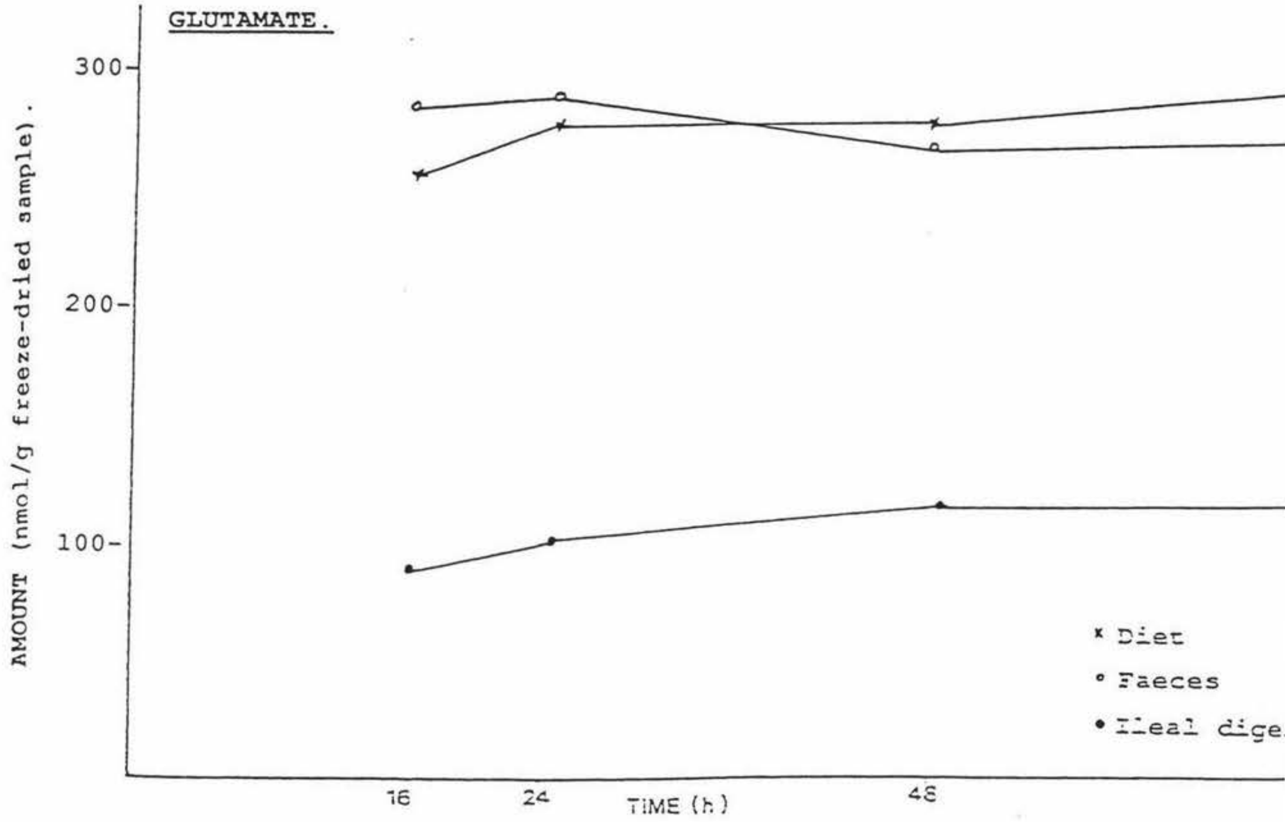


VALINE.
AMOUNT (nmol/g freeze-dried sample).
240-
200-
160-
120-
80-
40-
y = 0.28x + 143
y = 0.33x + 74
y = 0.40x + 46
× Diet
∘ Faeces
• Ileal digest
16
24
TIME (h)
48

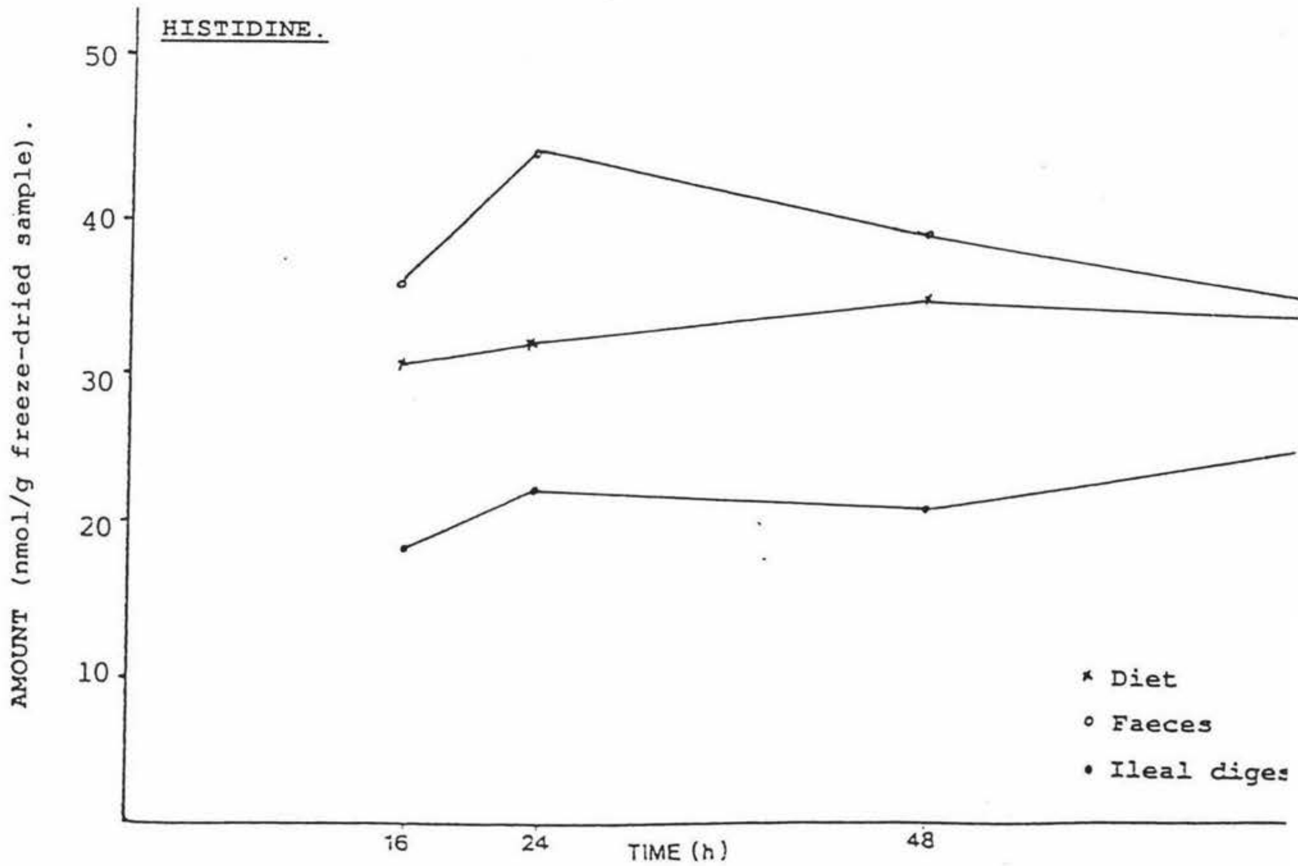
Curvilinear response.



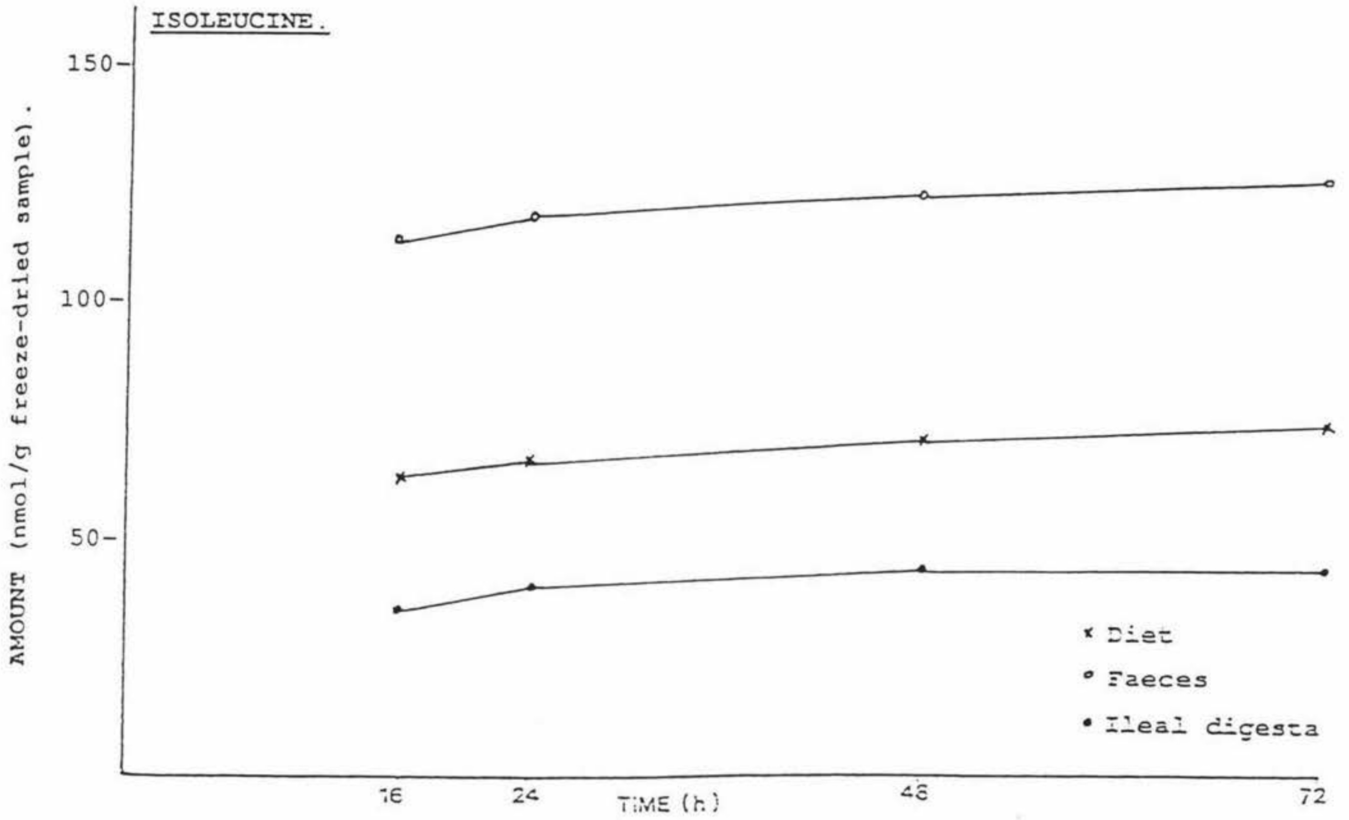


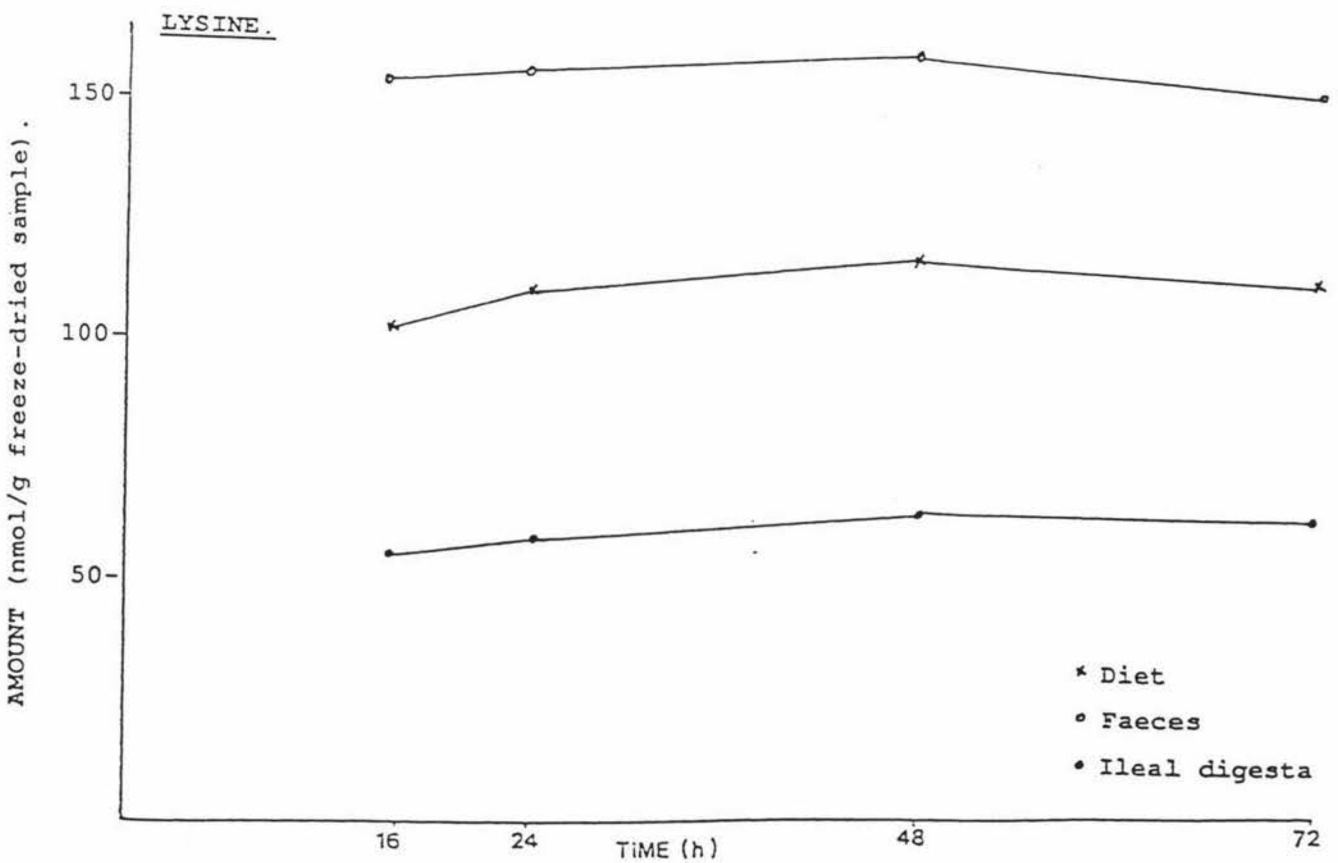


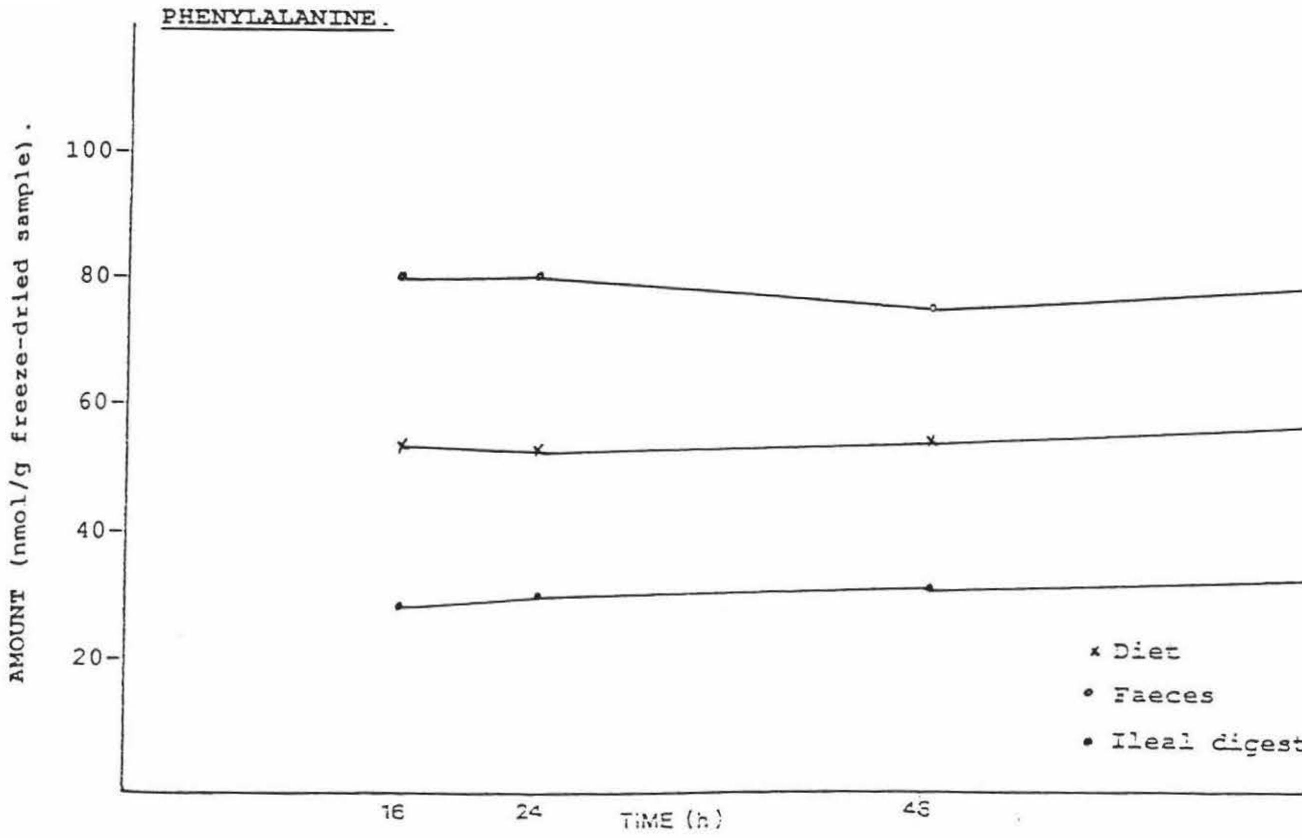
GLUTAMATE.
300
200
100
AMOUNT (nmol/g freeze-dried sample).
16
24
TIME (h)
48
Diet
Faeces

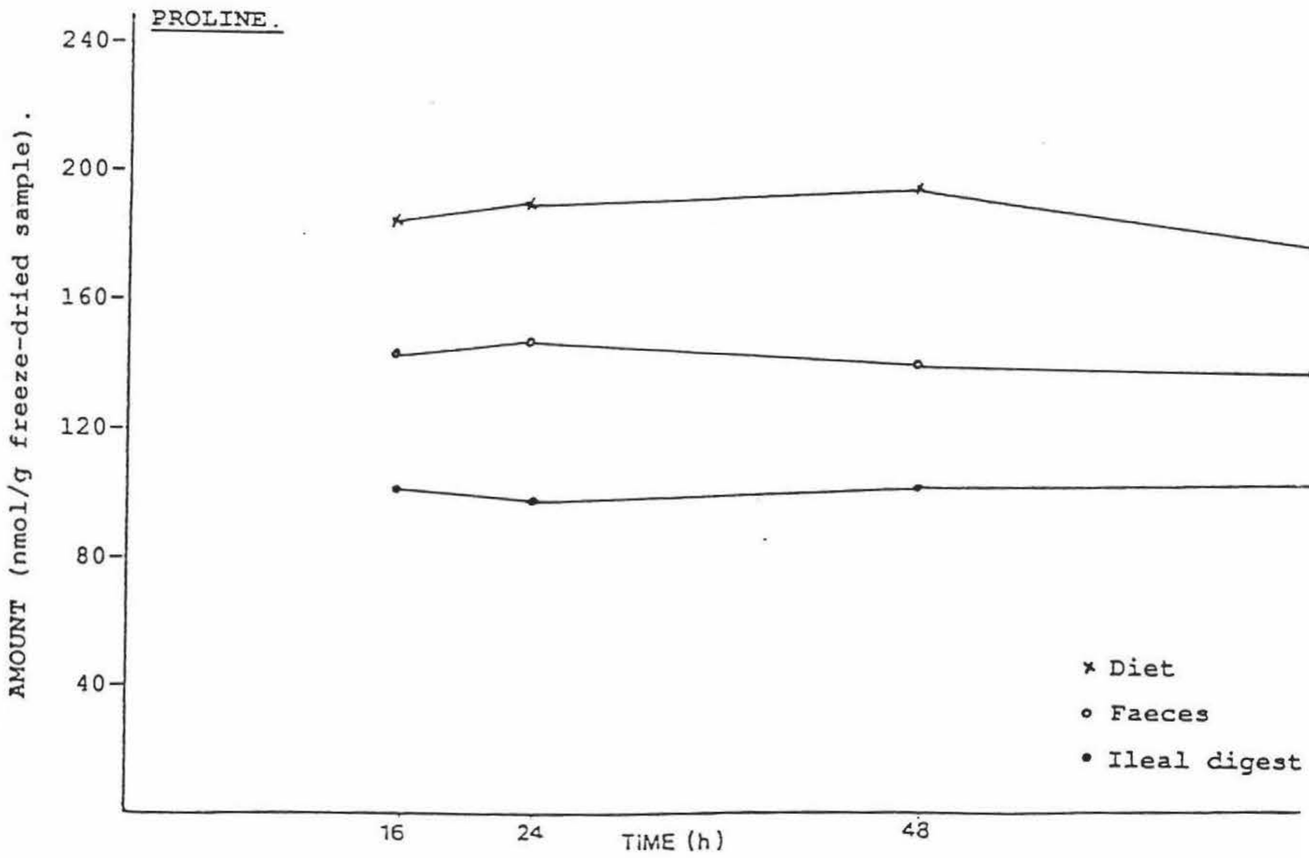


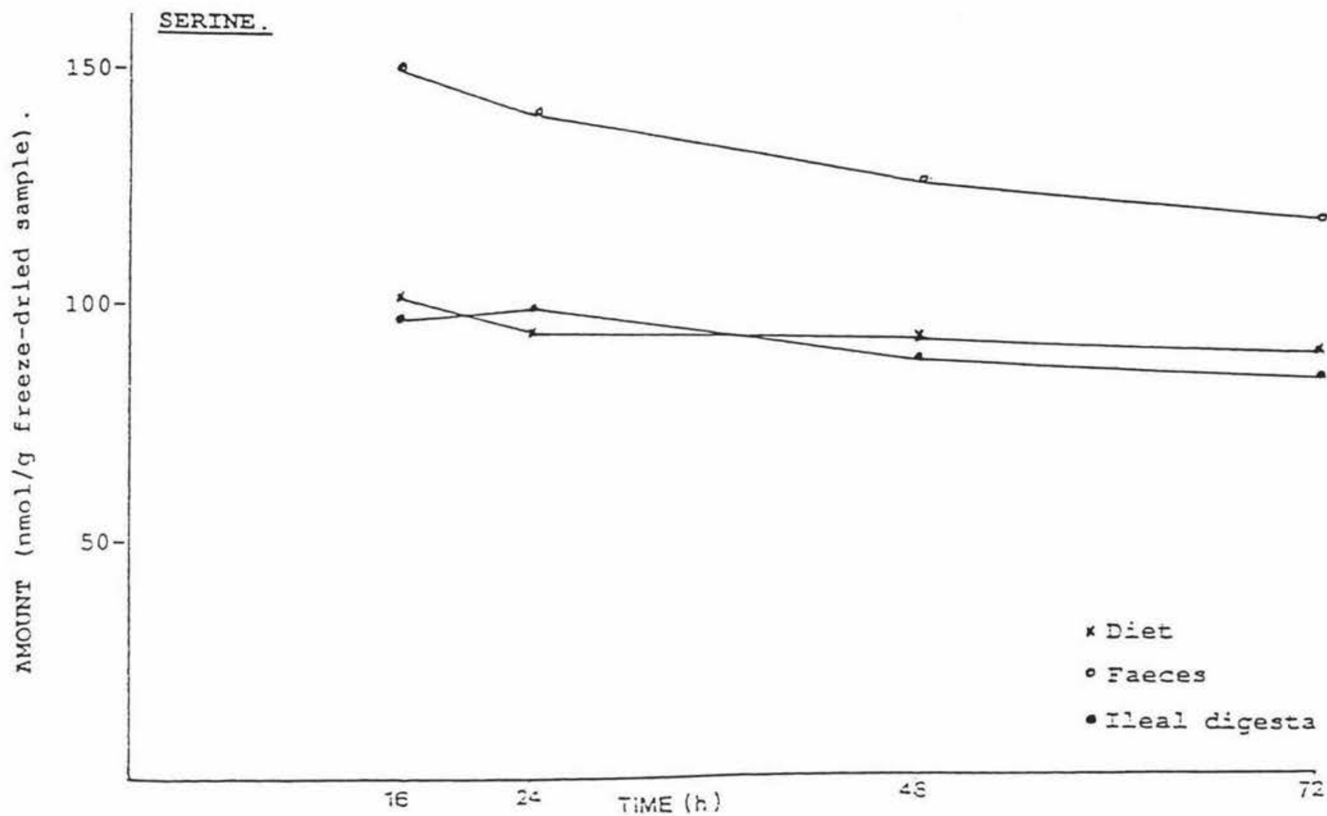
HISTIDINE.
50
40
30
20
10
AMOUNT (nmol/g freeze-dried sample).
16
24
TIME (h)
48
Diet
Faeces

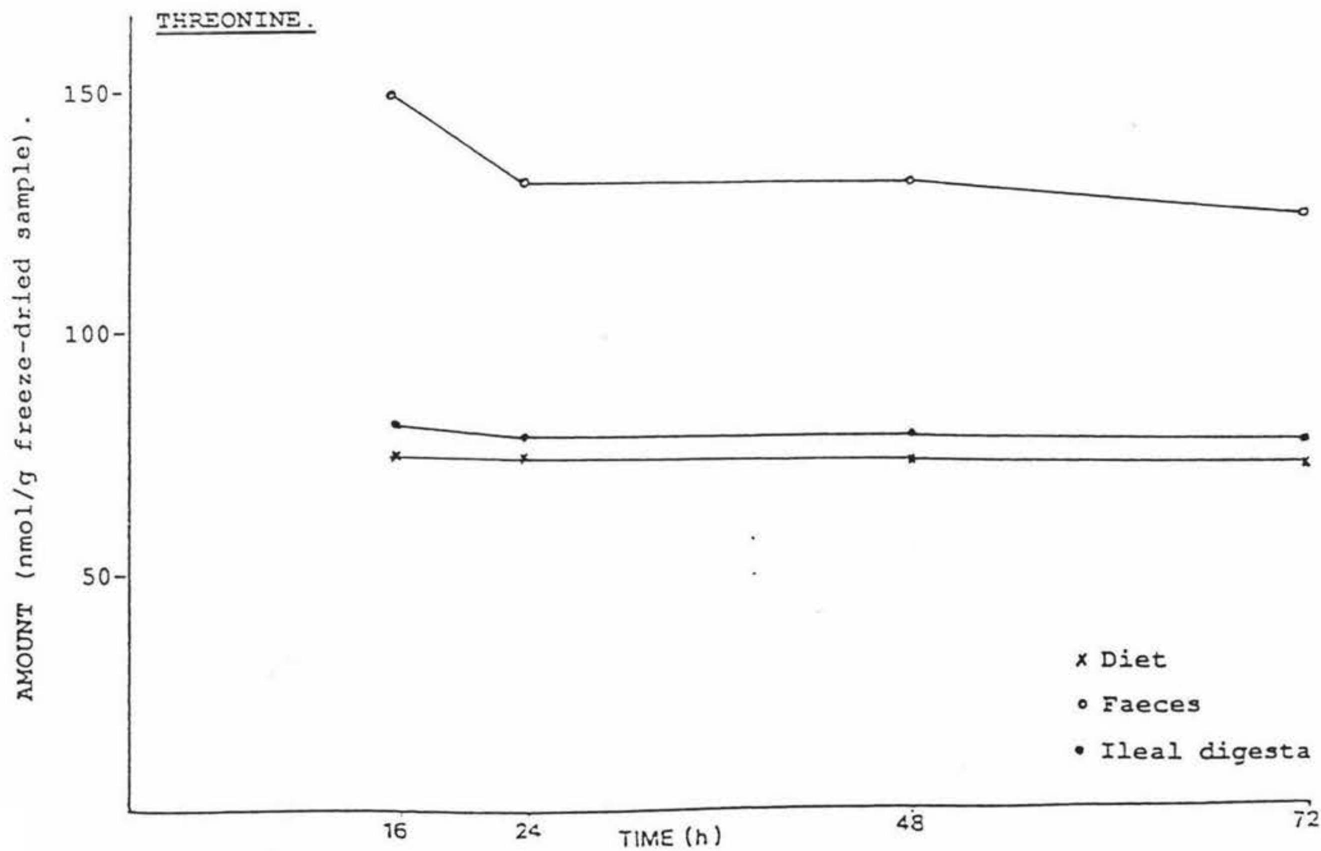

ISOLEUCINE.
150
100
50
AMOUNT (nmol/g freeze-dried sample).
Diet
Faeces
Ileal digesta
16
24
TIME (h)
48
72


LYSINE.
150
100
50
AMOUNT (nmol/g freeze-dried sample).
Diet
Faeces
Ileal digesta
16
24
TIME (h)
48
72


PHENYLALANINE.
AMOUNT (nmol/g freeze-dried sample).
100
80
60
40
20
16
24
TIME (h)
48
Diet
Faeces
Ileal digest


PROLINE.
AMOUNT (nmol/g freeze-dried sample).
240
200
160
120
80
40
16
24
TIME (h)
48
Diet
Faeces
Ileal digest


SERINE.
AMOUNT (nmol/g freeze-dried sample).
150-
100-
50-
x Diet
o Faeces
• Ileal digesta
16
24
TIME (h)
48
72


THREONINE.
AMOUNT (nmol/g freeze-dried sample).
150-
100-
50-
x Diet
o Faeces
• Ileal digesta
16
24
TIME (h)
48
72

APPENDIX 2.

2.1

Table 2.1. Example calculation to show the effect of the extent of faecal recovery of a non-digestible marker, on the determination of a coefficient of digestibility, refer Section 3.4.

Assumed AIA*1 recovery (%)	Calculated coefficient of digestibility*2	Faecal AIA*1 concentration (x_1)
50%	86.0	1.38
60%	88.3	1.65
70%	90.0	1.93
80%	91.2	2.20
90%	92.2	2.48
100%	93.0	2.75

*1 AIA = acid-insoluble ash.

diet AIA concentration = 0.26g/100g wet weight
diet dry-matter concentration = 42.08g/100g wet weight
faecal dry-matter concentration = 31.35g/100g wet weight
faecal AIA concentration = x_1

*2 $$\text{Digestibility} = \frac{\dfrac{42.08\ g/100g}{0.26\ g/100g} - \dfrac{31.35\ g/100g}{x_1\ g/100g}}{\dfrac{42.08\ g/100g}{0.26\ g/100g}}$$

2.2.

Conduct of a paired t-test for comparison of mean apparent dry-matter digestibility coefficients for adult humans given a mixed diet, determined by either total collection of faeces over 5 days or by reference to acid-insoluble ash (refer Section 3.4).

Hypothesis: H(d=0); Significance level = 5%

$$\text{t-statistic} = \frac{\text{mean difference (d)}}{\text{standard error of difference (S.E.d)}}$$

Subject	Digestibility coefficient		d	d^2
	Acid-insoluble ash	Total collection		
1	94.00	93.19	-0.81	0.66
2	92.86	94.14	1.28	1.64
3	94.18	94.46	0.28	0.08
4	89.53	90.70	1.17	1.37
5	92.38	93.56	1.18	1.39
6	93.00	93.78	0.78	0.62
			Ed 3.88	Ed^2 5.76

$$\text{mean difference} = \frac{3.88}{6} = 0.65$$

$$\text{standard deviation of d} = \sqrt{\frac{5.76 - (3.88/6)^2}{5}} = 0.81$$

$$\text{standard error of d} = \frac{0.81}{6} = 0.33$$

$$\text{t-value} = \frac{0.65}{0.33} = 1.97 \text{ (5 d.f.)}$$

Tabulated $t_{0.05} = 2.57$; Fail to reject the null hypothesis.

2.3.

Conduct of a paired t-test for comparison of the apparent dry-matter digestibility coefficients for six-week-old pigs given a mixed diet, by either total collection over 3 days or by reference to acid-insoluble ash, excluding pig#5 (refer Section 3.4).

Hypothesis: H(d=0); Significance level = 5%

Pig #1-4	Apparent digestibility of dry-matter		Σd	Σd^2
	Acid-insoluble ash	Total collection		
X$\pm$ S.E.	92.17$\pm$ 0.17	93.41$\pm$ 0.29	4.97	6.38

$$\text{mean difference} = \frac{4.97}{4} = 1.24$$

$$\text{standard deviation of d} = \frac{6.38 - (4.97)^2/4}{3} = 0.26$$

$$\text{standard error of d} = \frac{0.26}{4} = 0.13$$

$$\text{t value} = \frac{1.24}{0.13}$$

$$= 9.54 \text{ (3d.f.)}$$

Tabulated $t_{0.05}$ = 3.182. Reject the null hypothesis.

APPENDIX 3.

Table 3.1. Age, weight, sex and occupation of the subjects involved in the human study, refer Section 4.2.

Subject	Age (yrs)	Weight (kg)	Sex	Occuption
1	-*1	-*1	Female	-*1
2	42	56	Male	Optometrist
3	66	78	Male	Retired
4	42	67	Male	Clerical
5	-*1	-*1	Male	Owner/driver
6	46	86	Female	Secretarial
7	60	65	Male	Scientist
8	22	60	Female	Student
9	23	80	Male	Farmer
10	23	60	Female	Veterinarian
11	32	80	Male	Scientist
12	55	65	Female	Scientist

*1 Information not available.

Table 3.2. Daily gross energy supplied from sugar and lemonade for six ileostomates in the human study, refer Section 4.2.

Subject	Energy from sugar(kJ)	Energy from lemonade(kJ)
1	0	100
2	170	230
3	0	230
4	500	530
5	420	720
6	500	100

Table 3.3. Time spent on different activities as indicated by 11 subjects in the human study, and calculated daily energy expenditures, refer Section 4.2.

Subject	Sleep		Work		Exercise		Other		Total
	T (h)	E (kJ)	T (h)	E (kJ)	T (h)	E (kJ)	T (h)	E (kJ)	E (kJ)
2	8	2200	5*1	2000	2	1100	9	2500	7800
3	8	2200	4	3500	-	-	12	4230	9930
4	8	2200	8	3630	3	2230	5	1320	9380
5	8	2200	8	7300	1	600	7	2200	12300
6	8	2200	6	3220	2	1000	8	2400	8820
7	8	2200	8	3600	1	1080	7	2720	9600
8	8	2200	9	4100	1	1080	6	2800	10180
9	8	2200	10	11800	-	-	6	2000	16000
10	8	2200	8	2800	1	1000	7	2500	8500
11	8	2200	9	4050	-	-	7	4250	10500
12	8	2200	8	2880	-	-	8	4120	9200

*1 This subject had time off work during the trial.

Table 3.4. The daily quantities of sugar and lemonade for three alternative diets followed by six adult human subjects during the faeces collection of the human study, refer Section 4.2.

	DIET		
	A	B	C
		Amount consumed.	
Lemonade (ml)	250	375	500
Sugar (g)	10	15	20

APPENDIX 4.

Table 4.1. The mean ileal and faecal digestibilities (%) of the individual sugars of the pectin and hemicellulose fractions for humans (65kg) and pigs (25kg), refer Section 6.3.c.

Component	HUMAN		PIG	
	Ileal	Faecal	Ileal	Faecal
PECTIN				
Rhamnose	89.8	99.8	92.3	97.9
Arabinose	87.9	96.4	89.8	98.2
Xylose	87.5	96.0	93.8	98.4
Mannose	91.6	98.2	96.0	99.7
Galactose	91.9	98.4	92.7	99.6
Glucose	91.6	99.5	93.5	98.9
Uronic acids	70.0	94.9	81.2	95.9
HEMICELLULOSE				
Rhamnose	48.0	81.4	63.2	95.9
Arabinose	38.9	98.3	45.3	99.8
Xylose	54.1	98.5	41.1	99.8
Mannose	77.4	92.3	67.3	98.6
Galactose	26.8	91.0	75.7	98.5
Glucose	71.6	96.5	69.9	99.4
Uronic acids	74.8	97.0	66.5	97.7

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