Nutritional and Physiological Effects of Short-term Feeding of an Early Maillard Browned Casein to Growing Pigs

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

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ABSTRACT

In the present study the digestion of an early Maillard browned casein and the absorption and excretion of the digestion products, were evaluated in the growing pig. Additionally, the physiological responses to short-term ingestion were determined.

In an animal study male pigs were fed a lysine and glucose supplemented early Maillard browned casein at high levels (30 %) in a well balanced diet, for a period of 26 days. Further, a similar early Maillard browned casein was radioactively labelled with U^{14}C-glucose and fed to pigs to differentiate Maillard compounds *in-vivo* into early Maillard products (Fructosyl-lysine) and advanced Maillard products (residual radioactivity not attributed to fructosyl-lysine). Total urine and faeces were collected and on slaughter a variety of physiological samples removed.

The apparent ileal and faecal digestibility of nitrogen, amino acids and Maillard products (total radioactivity, ε-DF-L and residual radioactivity) were determined. Additionally, ileal digesta was chromatographed by Sephadex G-15 gel filtration. Heat treatment caused a significant ($P < 0.05$) decrease in the digestion of nitrogen, and several non-essential and essential amino acids at the terminal ileum. Acid hydrolysed lysine (AHL) and arginine decreased the most. Regenerated lysine (RL) from the acid hydrolysis of ε-DF-L, greatly influenced the acid hydrolysed lysine content in ileal digesta and the diet. Regenerated lysine and, therefore, the apparent ileal digestibility of actual lysine (AL = AHL - RL) was found to vary considerably depending on whether ε-DF-L was present as a free molecule or protein bound. However, calculation of ileal lysine availability by acid hydrolysis was a predictive measurement of the actual lysine availability. The significant decreases in apparent amino acid ileal digestibility was accounted for by the presence of small limit peptides, 2 - 3 residues in length (430 MW). These appeared to be associated with Maillard compounds, particularly ε-DF-L. The FDNB reactive lysine content in ileal digesta showed that the majority of actual lysine was present as N-terminally peptide bound. Microorganisms in the large intestine significantly ($P < 0.05$) digested amino acids from the terminal ileum to the extent where differences due to heat treatment in the faeces were not apparent. The largest differences between apparent ileal and faecal digestibility coefficients showed a high correlation (0.99) with the least apparent ileal digestibility coefficients - particularly for the digestion of Maillard products. Maillard

products were detected in the small intestinal mucosa and both portal and jugular serum. The excretion of Maillard products was similar to other reported studies. However, large proportions of Maillard products, apparently absorbed by the terminal ileum, remained unaccounted for. A tabulation of the dietary intake levels of Maillard products, their digestion, absorption and excretion are reported. There was no major change in the urinary excretion of amino acids from the heated diet. Sephadex G-15 chromatography of urine showed that the Maillard products, mainly ε-DF-L, eluted in a molecular weight region (715 MW) higher than that present in ileal digesta. The former had a low association with amino acids.

Physiological responses were determined over a short-term (26 day) period to determine the effect of the ingested Maillard products on protein digestive functioning and liver and kidney responses to their metabolic transit. There were no major physiological changes related to protein digestive functioning in pigs. The enzymatic activities of pepsin, chymotrypsin and trypsin remained the same in digesta and tissues. There appeared ($P < 0.1$) to be an increase in the aminopeptidase N activity and protein content at the terminal ileum. The relative organ weights of the stomach, small intestine, caecum and large intestine remained the same. However, significantly ($P < 0.05$) lighter pancreas and greater proportions of small intestinal mucosa were shown. Furthermore, there was a significant ($P < 0.05$) decrease in the stomach contents and increase in the small intestine contents. There were also no major physiological changes to the liver and kidney functioning in pigs, with jugular serum enzymatic activities (alanine transaminase, aspartate transaminase and γ-glutamyl transpeptidase) and the levels of blood urea nitrogen (BUN) and creatinine, and the BUN/creatinine ratio remaining unchanged. Furthermore, urine volumes, specific gravity, osmolality, the excretion of urea nitrogen (N) and creatinine, and the urea N/creatinine ratio remained the same. Some concern was raised over the colour of the urine being reddy-brown from pigs fed the heated diet, but clinical analysis tests (iron, bilirubin, urobilinogen, hexosamines, porphyrins, indicans and homogentisic acid) were negative and did not identify the nature of these pigments. They do not appear to be from a dietary origin.

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Chapter 1

INTRODUCTION

The Maillard, or non-enzymatic browning reaction describes the chemical reactions between the free amino and carbonyl groups in foods. These can lead to browning and flavour production. The reaction commonly occurs when proteins are heated in the presence of reducing sugars. This has been known for many years to decrease the nutritive value of the protein, particularly through the unavailability of lysine and the subsequent decrease in protein digestibility. However, reduction in nutritive value of browned proteins does not seem to be limited to either the loss or unavailability of amino acids. The ingestion of Maillard products can causes several physiological changes, some of which can be considered as being nutritionally related, whereas others have been suggested to be due to the presence of toxic compounds. In the present study digestion of an early Maillard browned casein, absorption and excretion of the digestion products, and physiological effects after short-term feeding were examined in relation to human nutrition, by using the growing pig as a model.

Man is the only species known to heat its food sources. As far as we know fireplaces age back 400,000 years. The fireplace not only provided warmth and sanctuary from the environment, but also increased the palatability and preservation of foodstuffs. As a consequence this increased the variety of consumable foods. These attributes became an important factor in the evolution of man. The chemical reactions responsible for the browning of foods through heat treatment were not uncovered until 1912, when a French scientist, Louise-Camille Maillard, described the formation of brown pigments from heating a solution of glucose and lysine. In his last report in 1917, Maillard discussed these findings in relation to the browning of foods with heat treatment. Subsequently, these non-enzymatic browning reactions were termed the Maillard reaction. For several reasons, including increased population pressure, unconventional food resources and more advanced processing technology, there has been an increase in the heat processing of foods and animal feedstuffs (Knipfel, 1981). In fact many of the protein components in our foods have been heat treated in some way. It was not until 1948 that

detrimental effects were noticed through a reduction in the nutritive values of proteins from the losses of certain amino acids, especially lysine (Patton *et al.*, 1948). The impact of this finding was not on domestic cooking methods, but drew attention to the heat processing and storage of protein concentrates such as fish, soybean and milk products. Research was then directed to alleviate the problems of food deterioration and to ensure that the manufacturing did not adversely effect nutritive values and, therefore, protein quality to significant degrees. Because of the important benefits of heat processing in the food industry and our own domestic cooking methods, for namely: development of flavour (roasting coffee and coco beans, baking breads and cakes, and cooking meats); colour; sterilization; and drying, it was necessary to study further the chemistry, nutritional effects and safety aspects of the heat treatment on foods. The evidence for inhibitory and anti-nutritive properties were first noticed by Rao *et al.* (1963) when supplementation of those amino acids lost could not completely restore the biological value of the protein. As a consequence more emphasis has recently been placed on the physiological effects of the ingested Maillard products which may cause these anti-nutritive properties.

1.1 The advantages and disadvantages of heating foods

A general summary of the advantages and disadvantages of heating food sources are presented in Table 1.1.

During the processing of foods various forms of heat treatment cause chemical reactions with a variety of amino acid side chains. The effects of these treatments on the nutritional and metabolic aspects of protein modification can be classed into four types of damage (Finot, 1983):

(1) Maillard reactions between free amino and carbonyl groups in foods.

(2) Isopeptide formation under severe conditions of heat treatment between lysine residues and glutaminyl and asparaginyl residues.

(3) Modification of amino acid side chains by alkaline treatment, e.g. lysine to lysinoalanine and cysteine to lanthionine.

(4) Oxidation in foods. Firstly, protein-polyphenol interactions by the oxidation

of polyphenols in various plant sources to reactive quinones. Secondly, the oxidation of the sulphur containing amino acids (cysteine and methionine) and tryptophan.

Since the main emphasis of this thesis is on Maillard reactions, the other types of damage are not discussed directly in the course of this investigation.

Table 1.1 *The advantages and disadvantages of heating foods.*

Advantages	Disadvantages
Chichester and Lee (1981).	*Lee et al. (1981).*
Destroys toxic constituents in foods: antagonists to vitamins, enzyme inhibitors (trypsin inhibitor) and other toxic proteins.	Decrease in the availability of amino acids, through destruction and a decrease in protein digestibility, particularly lysine.
Increases the digestibility of certain food sources by protein denaturation and breakdown of plant cell walls.	Formation of compounds which may have inhibitory or antinutritive properties.
Increases the palatability of foods by the production of aromas, colours and tastes.	Possible formation of toxic compounds.
	Formation of potentially mutagenic compounds in over-cooked foods.

1.2 Chemistry of the Maillard reactions

Most of the research into the chemistry of the Maillard reaction has involved the use of model sugar-amine systems (Hodge, 1953). This is necessary because of the complexity of reactions and the literally hundreds of different compounds formed. Many of the proposed pathways lead to products which have been identified in proteins and foods.

As mentioned previously the Maillard reaction describes the chemical reactions between the amino and carbonyl groups in foods. They are known to occur between aldehydes, ketones, oxidized fats and reducing sugars with amino acids, peptides and proteins. The first coherent scheme of the Maillard reaction was presented by Hodge (1953). A summary of the chemistry of the Maillard reactions have been reported (Mauron, 1981)

and discussed in relation to the reactions between amino acids and reducing sugars. A simplified representation is given in Figure 1.1, in which the reaction has been split into early, advanced and final Maillard reactions.

The early Maillard reaction involves the reversible attachment and condensation of the amino group and reducing group to form a Schiff's base which rapidly cycles to an aldosylamine. The unstable aldosylamine then undergos an irreversible transition (by the Amadori rearrangement) to a ketosylamine, otherwise known as the Amadori compound. Examples of Amadori compounds are lactulosyl-lysine and fructosyl-lysine, isolated from milk powders (Moller *et al.*, 1977 I, II & III). The mechanism presented by Hodge (1953) involving the Amadori rearrangement as the key step to the formation of brown pigments was accepted for over 25 years. A new 'early' mechanism leading to brown pigment formation, which may also be in operation, has been proposed (Namiki *et al.*, 1977), which involves sugar fragmentation and free radical formation, prior to the Amadori rearrangement. These free radicals were demonstrated (Namiki *et al.*, 1977) to interact as highly reactive precursors in the formation of brown pigments.

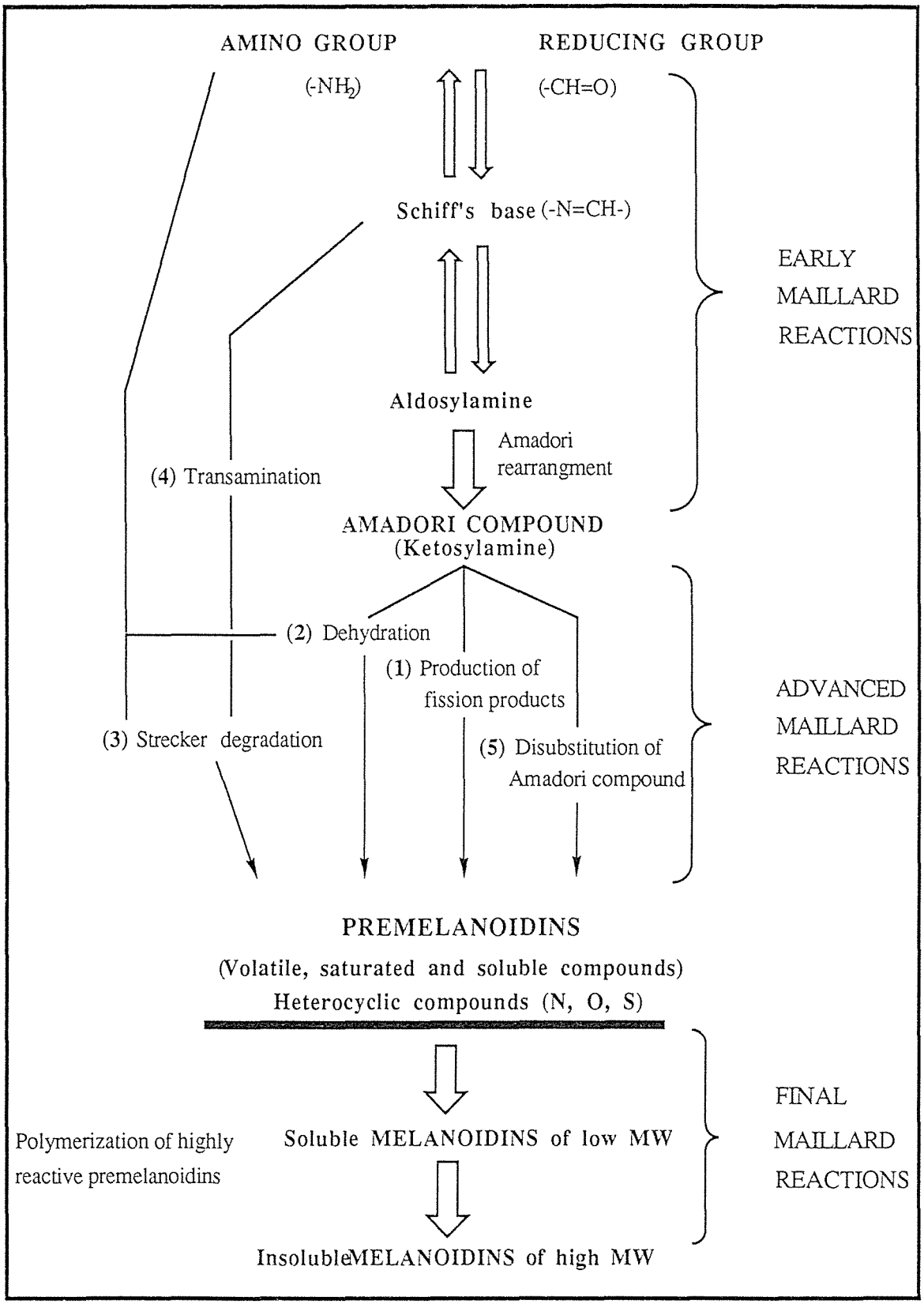
The Amadori compound is the central point from which most of the advanced Maillard reactions occur. Only five major pathways have been isolated in a very complex series of reactions which lead to volatile, unsaturated and soluble compounds. The literally hundreds of compounds formed may, in later stages of the advanced Maillard reactions, lead to the formation of the heterocyclic products responsible for the aromas and flavours of foods. These advanced Maillard products are also known as premelanoidins. The original definition of premelanoidins (Adrian, 1974) encompassed all soluble compounds formed in the Maillard reaction. However, the distinction is now made that they do not include early Maillard products and soluble melanoidins (Finot & Magnenat, 1981). It should be noted that the term advanced Maillard reactions has also been used in a broader definition which includes the final Maillard reactions described below (Finot & Magnenat, 1981).

The final Maillard reactions involve the production of brown pigments, ranging from soluble low molecular weight compounds to insoluble high molecular weight compounds. These result from the polymerization of highly reactive derivatives that are formed in the advanced Maillard reactions (premelanoidins). These form under more intense conditions of heat treatment and can be classified as mainly unsaturated nitrogen containing

compounds called melanoidins (Adrian, 1974). Melanoidins have been described as amphoteric polymers (Horikoshi & Gomyo, 1976) and heterocyclic amines are thought to be the major cause of the brown colour development (Mauron, 1981).

The Maillard reaction occurring in a protein may change its tertiary structure. When the ε-amino group of lysine reacts with a reducing sugar, the formed compounds will become peptide bound. However, other compounds such as N-heterocyclics may be free as these appear to be largely responsible for the roasted, bready or nutty aromas of foods, probably occurring by the interaction of reducing compounds with free amino acids in the food source. The protein bound early and advanced Maillard products may interact to form final Maillard products resulting in cross links within the tertiary structure, other than the polypeptide chain. Valle-Riestra and Barnes (1970) suggested that the production of melanoidins may cause further interactions with the protein to form enzymatically resistant cross linkages. Further information about these types of interactions was gathered by Namiki *et al.*, (1977). They proposed that if pyrazine type radicals are formed, their interactions would suggest the presence of new inter and intra-molecular cross linkages between two protein bound amino groups. Unfortunately, information on the structural relationships of the protein bound interactions is limited because of the complexity and variety of Maillard products formed.

Figure 1.1 *A simplified representation of the early, advanced and final Maillard reactions.*



1.3 Factors influencing the Maillard reaction

Free amino groups and carbonyl groups have varying susceptibilities to the Maillard reaction, dependant on the molecule from which it is donated. The most reactive amino groups are the free α-N terminal groups of amino acids, peptides and proteins (Adrian, 1974). The most reactive amino acid side chain is the free ε-amino group of lysine (Lea & Hannan, 1950 III). The remaining basic amino groups of arginine and histidine also react, but to a much lesser extent (Lea & Hannan, 1950 III). The pattern of amino acids (relative order and amount) in a protein has also been shown to influence the susceptibility to heat damage (Knipfel *et al.*, 1975). Of the sugars in foods, only the reducing sugars provide the necessary carbonyl group to react. Aldopentoses are the most reactive and aldohexoses react more than reducing disaccharides. The non-reducing sugars do not participate, although sucrose is hydrolysed to its constituent reducing monosaccarides (fructose and glucose) from heat treatment (Mauron, 1981).

The temperature and duration of heat treatment obviously effects the extent of the Maillard reaction. The intensity of temperature and its duration results in different proportions of early, advanced and final Maillard reaction products. This is demonstrated by different intensities of heat treatment producing a variety of aromas and flavours. In general at a given temperature the formation of brown pigments (melanoidins) augments with the square of reaction time (Mauron, 1981).

Moisture content is important in the Maillard reaction. This is demonstrated by the differences noticed from the boiling and steaming of foods (which does not form browning) compared with baking, roasting and frying (which does cause browning). Browning is not observed at moisture levels of 0 % or above 80 % (Wolfram & Rooney, 1953; and Lea & Hannan, 1949 I). While the maximum rate of lysine loss has been observed at a moisture level between 15 and 18 % (Lea & Hannan, 1949 I), maximum browning has been observed at a moisture level of 30 % (Wolfrom & Rooney, 1953; and Lea & Hannan, 1949 I).

The pH also influences the Maillard reaction, with alkaline conditions accelerating and acidic conditions inhibiting the Maillard reaction due to the protonation state of the free amino group (Lea & Hannan, 1949 I).

1.4 Nutritional aspects

In 1914 Osborne and Mendel demonstrated that the nutritive value of a protein was dependant on the amounts of its constituent amino acids, relative to the requirements of the organism for specific metabolic functions. The amino acid composition of proteins is adequate in the evaluation of the protein quality of highly digestible protein sources (Block & Mitchell, 1946; and Oser, 1951), but inadequate in poorly digestible proteins.

It is well documented that there is a reduction in the nutritive values of proteins heat damaged in the presence of carbohydrates (Henry & Kon, 1950; Schroeder *et al.*, 1951; Rao *et al.*, 1963; Miller *et al.*, 1965 III; Rios-Iriarate & Barnes, 1966; Nesheim & Carpenter, 1967; Osner & Johnson, 1968; Sgarbieri *et al.*, 1973; Adrian, 1974; Hurrell & Carpenter, 1974; Tanaka *et al.*, 1975 A; Tanaka *et al.*, 1977; Kimiagar *et al.*, 1980; Knipfel, 1981; and Tsen *et al.*, 1983). Nutritive values are commonly determined using biological assays, mainly using the rat as a model animal, such as protein efficiency ratios (PER), net protein retention (NPR), net protein utilization (NPU), relative nutritive value (RNV) and biological value (BV). These are direct measures of the utilization of protein in terms of either body weight (PER, RNV and NPR), or nitrogen (BV and NPU). Formulas used for the above calculations of nutritive values are given in Table 1.2 (A).

The subsequent reduction in the nutritive value of a protein heated in the presence of reducing sugars has been mainly attributed to a decrease in the availability of amino acids. The availability of amino acids can decrease for three major reasons (Knipfel, 1981): (1) The destruction of indispensable amino acids due to the formation of Maillard products which cannot be utilized; (2) A decrease in protein digestibility and, therefore, absorption; (3) The formation of absorbed but unavailable compounds which are finally excreted in the urine. The term availability defines those amino acids having the potential for protein synthesis, which are absorbed and utilized (Erbersdobler, 1976). The availability of amino acids has been reviewed by several workers (Erbersdobler, 1976;

Hurrell & Carpenter, 1977; and Austic, 1983). Knipfel (1981) has studied the effects of heat treatment on the protein and amino acid utilization by the monogastric animal and stated that in order to assess the nutritive value of processed proteins three factors must be considered:

(i) The total amount of amino acid ingested.
(ii) Relative proportions of amino acids (pattern).
(iii) Degree to which an animal can utilize the ingested material, i.e. digestibility and availability of the absorbed products.

The most immediate consequence of the Maillard reaction is the destruction of lysine and the concurrent formation of Maillard products. Damage to lysine and its significance has been reviewed by Carpenter and Booth (1973). A variety of Maillard compounds have been tested for their availability as lysine. True Schiff's bases (and aldosylamines as the reaction is reversible) of lysine have been shown to induce equivalent growth promoting effects to that of lysine and are, therefore, 100 % utilized (Finot *et al.*, 1977). In contrast, Amadori compounds have been shown not to be utilized (Finot *et al.*, 1977; Erbersdobler, 1976; and Plakas *et al.*, 1988), as have premelanoidin mixtures (Finot & Magnenat, 1981) and melanoidins (Finot & Magnenat, 1981; and Homa & Fujimaki, 1981). In mildly heat damaged proteins the reduction in nutritive value can usually be accounted for by lysine losses alone, whereas in the case of more severely heat damaged proteins the digestibility and availability of the protein is also considerably reduced.

The digestibility of heated damaged proteins have been commonly determined by the difference in amino acid or nitrogen content from the dietary intake compared to the output in the indigestible residue (Table 1.2 - B). Estimates of the effect of heat processing on the digestibility of proteins have commonly used the faecal material as the indigestible residue (Evans & Butts, 1949; Schroeder *et al.*, 1951; Mauron *et al.*, 1955; Miller *et al.*, 1965 II; Ford *et al.*, 1967; Valle-Riestra & Barnes, 1970; Adrian, 1974; Sgarbieri *et al.*, 1973; and Knipfel, 1981). However, accurate estimates of digestibility can only be determined on the digesta at the terminal ileum, as the micro-organism population of the large intestine can further digest material, leading to overestimates of digestibility (Varnish & Carpenter, 1975; Bayley *et al.*, 1974; Zebrowska & Buraczewski, 1977; Hurrell & Carpenter, 1978; Low, 1979; Austic, 1983; Moughan and Smith, 1984; and Sauer & Ozimek, 1986). In the two recent reviews (Austic, 1983 and Sauer & Ozimek, 1986) it

was concluded that the ileal analysis method was the most sensitive and accurate method for determining the amino acid digestibility in feedstuffs for pigs.

The decrease in apparent digestibility of Maillard heat damaged proteins can be attributed to three causes:
(1) The formation of enzymatically resistant cross linkages which cannot be hydrolysed *in-vitro* (Mohammed *et al.*, 1949; Evans & Butts, 1949; Lea & Hannan, 1951 IV; Ford & Salter, 1966; Moller *et al.*, 1977 I; and Clark & Tannenbaum, 1970 & 1973). These have been "proposed" to be also resistant to hydrolysis *in-vivo* (Buraczewski *et al.*, 1967; Boctor & Harper, 1968; Nesheim & Carpenter, 1967; Valle-Riestra & Barnes, 1970; Ford & Shorrock, 1971; and Mori & Nakatsuji, 1977).
(2) Indirectly from a contribution of amino acids from an endogenous origin due to (i) Maillard limit peptides competing for amino acid absorption sites (Buraczewski *et al*, 1967; Nesheim & Carpenter, 1967; Valle-Riestra & Barnes, 1970; Erbersdobler, 1976; Lee *et al.*, 1977 A; and Sherr *et al.*, 1989), and (ii) poorly digestible proteins enhancing losses of endogenously secreted proteins (Buraczewski *et al.*, 1967; Percival & Schneeman, 1979; and Schneeman & Dunaif, 1984);
(3) Maillard compounds have been shown to inhibit the digestion of free protein *in-vivo* (Adrian, 1974; and Oste & Sjodin, 1984) and inhibit certain proteolytic digestive enzymes such as carboxypeptidase A and aminopeptidase N *in-vitro* (Oste *et al.*, 1986). Adrian (1974) has also demonstrated *in-vitro* inhibition of the digestion of casein using a pepsin, trypsin, erepsin digestion mixture by the addition of free premelanoidins.

Absorbed but unavailable compounds may also contribute to the reduced nutritive value of the protein. This factor is not detectable by digestibility studies. Absorbed but unavailable lysine derivatives have been demonstrated in the urine of rats (Valle-Riestra & Barnes, 1970; Mori & Nakatsuji, 1977; and Liardon *et al.*, 1987) fed Maillard proteins. As mentioned previously the Maillard compounds are considered metabolically inert and, therefore, if absorbed are mainly excreted unchanged in the urine (Finot & Magnenat, 1981; and Erbersdobler, 1981). This mainly concerns lysine and its unavailability. Small proportions of absorbed but unavailable small peptides from severely heat damaged pure proteins have been reported in rat urine (Ford & Shorrock, 1971), which may contribute to the unavailability of amino acids.

Table 1.2 *A summary of the in-vivo measures of protein quality.*

(A)
DIRECT MEASURES: (These measures give a direct indication of utilization)

Protein efficiency ratios (PER) - *Osborne et al. (1919).*

$$PER = \frac{\text{gain in body weight (g)}}{\text{protein consumed (g)}}$$

Commonly assayed by a male rat growth trial over 4 weeks of a diet containing 100 g protein/kg diet fed ad libitum

Net protein ratio (NPR) - *Bender & Doell (1957).*

$$NPR = \frac{\text{(weight gain of test group + weight loss of non-protein group)}}{\text{weight of protein consumed}}$$

This is a form of PER but also takes into account the protein required for maintenance.

Biological value (BV) - *Mitchell (1924).*

$$\text{Apparent BV} = \frac{\text{N intake - (faecal N + urinary N)}}{\text{N intake - faecal N}} = \frac{\text{N retention}}{\text{N absorbed}}$$

Protein intake must be sufficient for adequate nitrogen (N) retention but must not be in excess of that required for maximum retention. True BV takes into account the obligatory losses of faecal and urinary nitrogen assessed from a protein free diet by subtracting them respectively.

Relative nutritive value (RNV) - *Hegsted et al. (1968).*
Varying protein intake levels (1 control and 3 tests) are fed for 4 weeks and the change in body weight measured and graphed against intake level. The slopes are related to the control as a percentage estimate.

Net protein utilization (NPU) - *Miller & Bender (1955).*
Product of the nitrogen digestibility calculated as described below and BV. It is a measure of the nitrogen intake which is retained.

(B)
INDIRECT MEASURES:

Digestibilities

Total collection

$$\text{Apparent} = \frac{\text{(total dietary N or aa intake - total sample N or aa excreted)}}{\text{total dietary N or aa intake}}$$

Sampling, using an indigestible marker (chromic oxide)

$$\text{Apparent} = \frac{\text{(total dietary N or aa/Cr - total sample N or aa/Cr)}}{\text{total dietary N or aa/Cr}}$$

This is a measure of what is apparently absorbed. True estimates need to take into account endogenous losses which are usually assessed by feeding a protein free diet. Digestibilities are commonly carried out on faecal material. Faecal residue has been affected by micro-organisms of the large intestine. Therefore, a more accurate estimate is on the digesta at the terminal ileum of the small intestine.

1.5 Toxicity

The reduced nutritive value of heated Maillard proteins does not seem to be limited just to the loss or unavailability of amino acids, as supplementation for these limitations did not completely restore the proteins quality, measured by its protein efficiency ratio (Rao *et al.*, 1963) and relative nutritive value (Sgarbieri *et al.*, 1973). Consequently, it has been suggested that products formed during the Maillard reaction may have inhibitory or antinutritive properties. The question of "toxicity" has arisen from the apparent physiological activeness of the ingested Maillard products and the adverse effects they appear to cause.

The ingestion of Maillard products over short-term biological assays of up to 3 months uncovered a variety of physiological changes in the rat (Adrian, 1974; Lee *et al.*, 1976; and Tanaka *et al.*, 1977). The physiological events noted were an initial diarrhea; a decrease in body weight; increases in the relative organ weights of the liver, kidneys and caecum; and increases in serum alkaline phosphatase, transaminases, glucose and urea nitrogen. These physiological responses have also been reported (Schwartz *et al.*, 1973) with rats on a reduced food intake. However, since these studies did not contain nutritionally equivalent diets, supplemented for the unavailability of amino acids caused by the heat treatment, the physiological responses are possibly related to nutritional factors.

With nutritionally equivalent diets, fed to rats over long-term assays of up to 12 months, it was shown (Kimiagar *et al.*, 1980) that as the feeding period increased the adverse effects become more pronounced being cumulative in nature and resembling that of toxic compounds. This study used 10 day browned egg albumin at an intake level of 10 %. with the browned and control diets, having protein efficiency ratios equal to 1.1. The higher levels of serum transaminases, alkaline phosphatase, blood urea nitrogen, urine specific gravities and the enlargement of some organs (liver, kidney, caecum and stomach) with the ingestion of the Maillard browned protein appear mainly indicative of liver and kidney damage from the adaptation to the processing of indigestible material.

Conversely, in a recent study (Pintauro *et al.*, 1983), results indicate that, after eighteen months of feeding a browned egg-albumin to rats, all reported anthropometric, clinical

biochemical and histopathological changes in the could be attributed to nutritional and/or dietary related factors. Forty day browned egg albumin was used in this study at a level of 3 % of the diet. The browned and control diets were of equal and high protein quality with protein efficiency ratios above 2.0. They attributed the findings by Kimiagar *et al.* (1980) to be nutritionally related and not to the ingestion of browned compounds, since the protein efficiency ratios were low and all their reported physiological responses were similar to the restricted food intake reported by Schwartz *et al.* (1973). However, it was recognized that the two studies contained quantitative and qualitative differences in the Maillard compounds present and that these differences may elicit dissimilar physiological responses.

A recent study (O'Brien & Walker, 1988) of the effect of dietary glutamate/glucose Maillard reaction products on the rat found a variety of physiological responses which were considered to be potentially toxic (severe diarrhea, significantly depressed body weights, dose related caecal enlargement, increases in the relative kidney and liver weights, significantly more pronounced cortico-medullary nephrocalcinosis, significant increase in urothelial thickness and an increase in the caecal goblet cell density). Their results indicate physiological adaptation to the metabolic transit of Maillard reaction products after 5 weeks of feeding to rats.

1.6 The present study

The simple pleasure of cooking and eating foods prepared in different ways has guided man to numerous methods and food sources. As heating is such an important factor in our eating habits it is necessary to evaluate the nutritional, physiological and safety aspects of the ingested Maillard products, in relation to human nutrition. In the past the majority of biological assays have involved the use of Sprague Dawely male rats as the experimental model. However, pigs resemble humans in more ways than any other non primate mammalian species because of anatomical and physiological similarities (Pond & Houpt, 1978) and has been commenly used as a laboratory animal (Mount & Ingram, 1971). The use of the pig as a model for human nutrition has been recently reviewed (Miller & Ullrey, 1987).

Proportions of early and advanced Maillard products and their consumption in the human diet is uncertain. Chapter 3 describes the production and analysis of an early Maillard browned protein, from heating a model casein-glucose mixture, with the aim of producing a realistic balance of early and advanced Maillard products.

It is well known that the major factor for the reduced nutritive value of heated proteins is the decrease in the availability of amino acids, through their destruction and decreases in digestion and absorption (Knipfel, 1981). However, the more accurate estimation of digestion using the ileal technique has been poorly investigated with Maillard proteins. There also appears to be lack of evidence for the ileal digestibility of Maillard products. The associated reduction in digestibility of the Maillard protein has been mainly attributed to limited proteolysis, by the formation of enzymatically resistant linkages and, therefore, the production of limit peptides. This has been well documented *in-vitro* but remains to be clearly investigated *in-vivo*. Of further interest is that certain Maillard products have been suggested to act as an absorption delayed material which are finally excreted in the urine (Mori, 1978). In view of the above, their has been no complete account of the digestion of an early Maillard browned protein and the absorption and excretion of its digestion products. Chapter 4 focus on these areas in relation to the reasonings for a reduced nutritive value, by feeding the early Maillard browned casein to growing pigs.

Physiological responses to the short-term feeding of Maillard browned proteins remains to be clearly investigated when considering the recent studies (Kimiagar *et al.*, 1980; Pintauro *et al.*, 1983; and O'Brien & Walker, 1989). In Chapter 5, the physiological responses indicative of digestive functioning and liver and kidney responses to the short-term ingestion (26 day) of the early Maillard browned casein were evaluated in a nutritionally adequate diet fed to growing pigs.

Finally in Chapter 6, an overview of the consequences of the Maillard reaction in light of evidence in Chapters 3, 4 and 5 are discussed with reference to future directives.

Chapter 2

CHEMICALS AND METHODS OF ANALYSIS

This Chapter specifically details all of the chemical and standard methods of chemical analysis used in the subsequent Chapters.

2.1 Chemicals

Chemicals used in this study were obtained from the following companies:

Sigma Chemical Company, St. Louis, USA:

α-D(+)Glucose (anhydrous), L-leucine, L-lysine, L-aspartate, L-alanine, glutathione, urea, bovine serum albumin, α-ketoglutarate, glycyl-glycine (free base), NADH, β–NADP (monosodium salt, yeast), hippuryl-L-phenylanaline, pyridoxal-5-phosphate, L-γ-glutamyl-3-carboxy-4-nitroanilide, N-benzoyl-DL-arginine-*p*-nitroanilide, N-succinyl-L-phenylalanine-*p*-nitroanilide, L-alanine-4-nitroanilide, trypsin, hexokinase (180 U/mg), glucose-6-phosphate-dehydrogenase (300 U/mg), enterokinase (3.9 U/mg), malic dehydrogenase porcine heart (850 U/mg) and lactic dehydrogenase type XXIX (1000 U/mg).

BDH Chemicals Ltd, Poole, England:

2,4-dinitro-1-fluorobenzene, ε-N-dinitrophenyl-lysine, ethylchloroformate, 3,5-dinitrosalicylic acid, chromium (III) oxide, and haemoglobin (bovine, technical powder).

Amersham, USA:

D-(U^{14}C)-glucose (230 mCi/mM) 37 MBq. 1 mCi, Nuclear Chicargo tissue Solubilizer (NCS) and 1-^{14}C-hexadecane (61 mCi/mM).

Pharmacia Fine Chemicals, Uppsala, Sweden:

Sephadex G-15 superfine gel matrix.

United States Biochemical corporation, Cleveland, Ohio, USA:

Tris base.

Serva:

Adenosine-tri-phosphate (disodium salt).

Tasman Vaccines Ltd., Auckland, New Zealand.

Tasmix, pig creep vitamin trace mineral premix.

Dairy Research Institute, Palmerston North, New Zealand.

Sodium caseinate powder and lactic-acid precipitated casein.

South Island Chemical Limited, Christchurch, New Zealand.

Pentabarbitone [Pentabarb 300 (300 mg/ml)].

Miles Labs, Mulgrave, Victoria, Australia.

Multistix 2820 A, Ames.

All other chemicals were of laboratory grade.

2.2 Methods of analysis

2.2.1 Measurement of available lysine

The influence of heat treatment on the availability of lysine was determined using the direct 1-fluoro-2,4-dinitrobenzene (FDNB) reactive lysine procedure of Carpenter (1960) as modified by Booth (1971). This technique uses the reaction of FDNB with the free amino groups in a protein, its hydrolysis and the extraction of the dinitrophenol-lysine (DNP-L); a yellow derivative which absorbs strongly at 435 nm. FDNB reactive lysine was also determined in ileal digesta samples to distinguish the proportion of lysine which was either free or N-terminally bound from that which was C-terminally or internally bound in peptides.

Typically 300 mg of sample was weighed into a 250 ml round bottomed flask. To this 10 ml of 1 M $NaHCO_3$ was then added and the sample gently shaken until fully wetted. Several glass balls along with 15 ml of FDNB solution (prepared by dissolving 0.4 ml of FDNB in 15 ml of ethanol) were added and the solution gently shaken for 3 hours with constant swirling to ensure an even dispersion. The ethanol was evaporated off using a heating isomantle until the sample had lost 12.5 g in weight.

Once the mixture had cooled to room temperature, 30 ml of 8.1 M HCl (giving a final concentration of 6 M), was slowly added followed by a few boiling chips. This was gently boiled under reflux for 16 hour on a heating isomantle. The hydrolysate was filtered while still hot through three pleated layers of Whatman No. 541 into a 250 ml volumetric flask. Residual material was quantitatively transferred and filtered using hot water. Once cooled the filtrate was made to volume with water.. A precipitate of dinitrophenol was allowed to settle by standing for 1 hour. Aliquots (2 ml of filtrate) were then transferred into each of two 15 ml graduated test tubes A and B.

The contents of A were extracted 3 times with 5 ml of peroxide free diethylether (DEE) (Prepared as in Vogel, 1948). As much of the DEE (the top layer) as possible was discarded using a pasteur pipette attached to a vacuum water pump. Residual ether was removed by placing the tube in a hot water bath (in the fume hood) until no more effervescence was noticed on gently shaking. The contents (once cool) were made up to 10 ml with 1 M HCl.

The contents of B were extracted once with DEE; residual ether was removed as before. Two drops of phenolpthalein indicator solution (400 mg/L of 60 % ethanol.) was added followed by 1 M NaOH until the solution changed to a pink colour. Then 2 ml of carbonate buffer (0.93 M $NaHCO_3$, 0.038 M Na_2CO_3, pH 8.5) was added followed by 0.05 ml of ethyl chloroformate (ECC) under a fume hood. The tube was firmly stoppered, vigorously shaken, and left to stand for 8 minutes. Concentrated HCL (0.75 ml) was very slowly added with gentle shaking. The contents were extracted four times with DEE as described above. Care was taken in the first extraction because of a pressure build-up. Once cool the contents were made up to 10 ml with water.

The absorbances of both A and B were read against water at 435 nm. Tube B was the blank and the difference between the two was attributed to the absorbance of the DNP-L.

Samples were assayed in duplicate. Runs were carried out in batches of 10, each containing a control sample of sodium caseinate. Additionally, the extraction step was monitored using duplicate 2 ml aliquots of a DNP-L standard solution prepared as follows: Approximately 314 mg of DNP-L was accurately weighed and dissolved in 250 ml of 8.1 M HCl overnight. Finally 10 ml was diluted to 100 ml with water and a 2 ml aliquot used in the extraction step. This solution when diluted 1/5 has an approximate absorbance at 435 nm of 0.4, this should be the same after the extraction steps.

Results were expressed as g lysine/16 g nitrogen, calculated as follows:

$$\text{g lys/16 gN} = \frac{Ws \times Au \times V \times 100 \times 10}{Wu \times As \times a \times CP}$$

Ws = weight of the standard in mg/2 ml; 0.1 if prepared as recommended.
Wu = weight of sample in mg.
As = nett absorbance of standard.
Au = nett absorbance of unknown.
V = volume of filtered hydrolysate (250ml).
a = aliquot of filtrate; 2 ml recommended.
CP = crude protein content per 100 g of material.

Values were multiplied by a factor of 1.09 to correct for the DNP-L loss during acid hydrolysis.

As FDNB reactive lysine was also carried out on ileal digesta the influence of the chromic oxide indigestible marker on FDNB values were evaluated as chromium has been demonstrated to reduce DNP-L values (Booth, 1971). This was carried out using Na-caseinate with and without chromic oxide added in the same concentrations as that in ileal digesta. No differences were shown.

2.2.2 Glucose determination

Free glucose was estimated in the heated casein-glucose mixtures to determine the proportion of glucose lost or bound (as Maillard products) by comparison with the non-heated control .

Sample preparation

Casein-glucose samples (1 g) were accurately weighed into a 50 ml beaker, suspended in 40 ml of water at pH 7.0 with gentle stirring. This was transferred to a volumetric flask and made up to a volume of 50 ml. The protein was isoelectrically precipitated at pH 4.5 by the addition of 1 M HCl. An aliquot of the supernatant spun at 3,000 rpm in a bench-top centrifuge for 5 minutes. The supernatant was assayed for glucose by the following procedures.

Jugular serum was assayed on the serum supernatants after centrifugation.

Dinitosalicylic acid method

Reducing sugars were determined following the method of Bernfield (1955), based on the reaction of a salicylic acid derivative with the reducing group of a sugar. The complex formed absorbing strongly at 580 nm.

DNS reagent (2 ml) was incubated with 1 ml of sample in a boiling water bath for 15 minutes. After the mixture had cooled 3 ml of distiled water was added and the absorbance recorded at 580 nm. The procedure was calibrated using a standard of 10 µmoles glucose/ml in the range of 0 -10 µmoles. Sample batches were run with a 5 µmole/ml standard as a check.

Direct hexokinase method

Glucose was determined according to the method of Nesse (1982) using the principle of coupling the conversion of glucose to glucose-6-phosphate by hexokinase to the reduction of $NADP^+$ to NADPH by glucose-6-phosphate dehydrogenase.

The following solutions were made: (1) Tris buffer (0.1 M, pH 7.5); (2) Enzyme reagent mixture (6 mM Mg^{2+} ; 1 mM ATP; 1 mM $NADP^+$; 0.75 kU/L hexokinase; and 0.75 kU/L glucose-6-phosphate dehydrogenase (G_6PDH); in Tris buffer); (3) Glucose standards (1 g/L; and 6 g/L).

The enzyme reagent mixture (2 ml) was pipetted into a 3 ml cuvette followed by 0.1 - x ml (sample volume) of 0.1 M Tris buffer . The absorbance was zeroed at 339 nm before the addition of sample. The effect of the sample on the blank absorbances was checked by reading separately in the same volumes of Tris buffer. Sample (x ml) was added and the absorbance recorded at 339 nm after exactly 10 minutes. A standard curve was constructed in the range of 0 - 100 ug using the 1 g/L glucose stock solution. Each batch of samples was run with a blank and a linearity check of 600 µg using the 6 g/L glucose standard.

2.2.3 Amino acid analysis

All hydrolysates were analysed with a Beckman amino-acid analyzer 119BL using a three buffer citrate system; buffer A (pH 3.53, 0.2 N Na^{2+}), buffer B (pH 4.12, 0.4 N Na^{2+}), buffer C (pH 6.4, 1.1 N Na^{2+}) on a Beckman spherical Type W-2 resin. The run time was extended on Buffer C to detect any furosine (acid hydrolysed product of ε-DF-L), which elutes between arginine and homoarginine. Both channels were run at an absorbance of 540 nm on scales of 0.5 and 0.1. Chromatograms were analysed using a Spectra-physics integrator (SP-4290). All analyses carried out in duplicate to an accuracy of $\leq \pm 3\%$. A Beckman calibration mixture (BCM), containing 25 nmol of each amino-acid, was run at the beginning of each batch.

Processing

Most samples did not require processing, except freeze-drying to a powder. In some cases with fluid samples an aliquot was dispensed into preweighed hydrolysis tubes, dried under vacuum, weighed and hydrolysed as described below.

Jugular blood and portal blood were deproteinized as follows: A 2 ml aliquot of sample was pipetted into a plastic centrifuge tube containing 8 ml of 10 % Trichloroacetic acid (TCA) and the sample vigorously shaken. After standing for 15 minutes the sample was centrifuged at 15,000 g for 10 minutes and a 4 ml aliquot of the supernatant transferred to a preweighed hydrolysis tube. The TCA salt was removed using 4 successive diethylether extractions; discarding the top layer. Residual ether was removed using a hot water bath. The sample was freeze-dried, weighed and hydrolysed as described below.

Hydrolysis

Sealed tube hydrolysis (Moore & Stein, 1963; Davies & Thomas, 1973).

All samples were processed according to this method. Typically 20 mg (maximum value) of each sample was weighed into a 16 X 15 cm Kimax test-tube and 2 ml of 6 M HCl (containing 0.1 % phenol) added. The tube was evacuated (degassing the sample) and sealed. This was hydrolyzed under reflux in an oven at 110 °C for 24 hours, after which the tubes were cracked and dried under vacuum over NaOH and P_2O_5. The residue was suspended in starting buffer A, transferred to a 1.5 ml Eppendorf microfuge tube, and the insoluble residue spun down at 12,000 rpm for 2 minutes. An appropriate aliquot was then loaded onto the Beckman amino acid analyzer.

2.2.4 Total nitrogen content

The nitrogen content of all materials were determined using an automated Kjeldahl technique (A.O.A.C., 1975). The Tecator Kjeltec auto 1030 analyzer was set up for macro and micro analysis depending on the nitrogen content and sample size.

Macro system: Samples were accurately weighed into large 250 ml Tecator digestion tubes to which 1 macro special kjel tab S 3.5 (containing 3.5 g potassium sulphate and 0.0035 g of selenium) and concentrated H_2SO_4 (10 ml) was added. The samples were digested on a Tecator 1015 digester for 1 hour or until colourless. These were cooled, 30 ml of water added and the sample placed in the machine for analysis.

Microsystem: Samples were accurately weighed into 100 ml tubes to which 1 micro kjel tab (containing 1.5 g potassium sulphate and 0.0075 g of selenium), to which concentrated H_2SO_4 (4 ml) was added. The samples were digested on a Tecator 1009 digester for 1 hour or until colourless. These were cooled, 10 ml of water added and the sample placed on the machine for analysis.

The analyzer was blanked using water until a constant value was obtained. The percentage nitrogen in the samples was calculated from the following equation:

$$\% N = \frac{14.01 \times M \times 100}{\text{mg sample}} \times (\text{ml titrant} - \text{ml blank})$$

M = Molar concentration of acid: Macro, 1.0 M; Micro, 0.5 M

2.2.5 ^{14}C Radioactivity measurements

All radioactive samples were analysed on a liquid scintillation spectrophotometer (Beckman LS 8000) under conditions appropriate for ^{14}C counting. All samples were counted in 15 ml of a toluene based scintillant appropriate for emulsion counting (PPO 4 g; POPOP 100 mg; TX-100 333 ml; and Toluene 667 ml). An internal standard (IS) technique was employed for quenching and efficiency determination using a known quantity of 1-^{14}C Hexadecane. Since the true counts of the internal standard are known the efficiency of counting in that sample can be calculated as follows:

$$\text{Efficiency} = \frac{\text{cpm (sample + IS) - cpm (sample)}}{\text{known dpm of IS}}$$

The sample counts were therefore corrected accordingly.

Sample preparation

Many of the radioactive determinations were on samples of a complexed biological nature, most being insoluble in normal scintillant mixtures. Therefore, a tissue solubilizer NCS (Nuclear Chicago Solubilizer) was used in conjunction with two major references (Lindsay & Kurnick, 1969; Sisenwine & Walkenstein, 1969; and the NCS bulletin (1987) on its applications). The following three digestion procedures were used depending on the type of sample. Preliminary work was undertaken to ascertain the maximum possible amount of sample soluble in 1 ml of NCS.

(I) Fluids (Blood and urine samples):

Aliquots (100 µl) were digested using 1 ml of NCS and 1 drop of water in a scintillation vial at room temperature for 24 hours.

(II) Soluble solids (diet and mucosa):

Approximately 50 mg of sample was accurately weighed into a scintillation vial. The sample was fully wetted with 0.15 ml of water and digested with 1 ml of NCS at 50 °C for 24 hours or until completely dissolved.

(III) Partially soluble solids (digesta and faecal samples):

Approximately 50 mg of sample was accurately weighed into a 1.5 ml Eppendorf centrifuge tube. The sample was fully wetted with 0.15 ml of water and digested with 1.0 ml of NCS at 50 °C for 24 hours with regular stirring. Toluene evaporated during this procedure and was, therefore, topped up to the mark after mixing. The tubes were

spun at 12,000 rpm for 10 minutes in an Eppendorf centrifuge and the supernatants transferred to vials. Residual material was resuspended in 0.5 ml of toluene, spun and the supernatant transferred as before.

Those materials which did not require solubilizing with NCS such as eluates from fractionated samples were dispensed into vials, freeze-dried and counted in 15 ml of scintillant.

Chemiluminescence

Since NCS generates high chemiluminescence, several precautions were taken:
(i) Glacial acetic acid 0.034 ml/1 ml NCS was added to each scintillation vial after digestion; this neutralizes the solution to pH 6 - 7.
(ii) Then 15 ml of scintillant was added after digestion in NCS and the mixture heated at 50 °C for 3 hours; this speeds up any residual chemiluminescence reaction
(iii) Vials were finally stored in a dark cupboard overnight before counting.

2.2.6 Chromium content

The chromium content of the diet, gastrointestinal contents and faecal samples were assayed following a revised method by Fenton and Fenton (1979). This uses the principle of oxidizing Cr^{3+} (green) under acid conditions to dichromate Cr^{6+} (yellow-red) which absorbs strongly at 435 nm.

Samples (4 - 6 g) were weighed into a preweighed dry 30 ml pyrex beaker and dried in an oven at 105 °C for 16 hours. The beakers were cooled in a desiccator and weighed for dry-matters. These were then ashed in a furnace at 500 °C for 24 hours, cooled in desiccators and reweighed for ash weights. Then 20 ml of the digestion mixture (150 ml water, 150 ml conc sulphuric acid; 200 ml 70 % perchloric acid and 10 g of sodium molybdate added last) was added and the mixture heated to 300 °C, on a Gallenkamp heating block, until a yellow-red colour developed. The mixture was heated for a further 15 minutes, cooled, quantitatively transferred to a 100 ml volumetric flask and made to volume with water. An aliquot was transferred to a 15 ml centrifuge tube and spun at 2,000 rpm for 5 minutes. The absorbance of the supernatant was measured at 440 nm against water using an Ultraspec II spectrophotometer with autofill II (Sci Med LKB Biochrom).

Samples were analysed in duplicate in batches of 60. Each batch also contained a series of chromic oxide standards. Two standard curves were constructed (from the ashed weights of chromic oxide), the range of which depended on the expected concentration of chromium in the samples, either 0 - 15 mg or 0 - 100 mg.

2.2.7 Enzyme assays

All of the following enzyme assays were monitored using a Gilford dual beam spectrophotometer (model 260) with recorder (Seckonic SS 250F). Four separate samples were assayed simultaneously at the appropriate temperature using a thermostatically controlled cuvette holder.

Chymotrypsin and trypsin

Chymotrypsin and trypsin in the ileal digesta and pancreas homogenate supernatants were assayed according to the procedures of Gieger (1984) and Gieger and Fritz (1984), following the principle of hydrolysis of N-succinyl-L-phenylalanine-*p*-4-nitroanilide (SPNA) and N-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) substrates respectively, with the release of 4-nitoanaline which absorbs at 405 nm (Erlanger *et al.*, 1961).

Chymotrypsin The following solutions were made: (1) Tris buffer (0.05 M, pH 7.6 containing 0.02 M $CaCl_2$); (2) Substrate solution SPNA (0.87 mM, pH 7.6 in Tris buffer). SPNA (10 mg) was dissolved completely in 1 ml of ethanol with gentle warming, made to 25 ml with Tris buffer and stored in the dark at 30 °C. This solution was made fresh daily.

Trypsin The following solutions were made: (1) Tris buffer (0.05 M, pH 8.2 containing 0.02 M $CaCl_2$); (2) Substrate solution BAPNA (0.8 mM, pH 8.2 in Tris buffer). BAPNA (11 mg) was completely dissolved in 0.25 ml of dimethylsulphoxide (DMSO), made to 25 ml with Tris buffer and stored in the dark at 30 °C. This solution was also made fresh daily.

The substrate solution (2.5 ml) was added, with 0.5 ml - x ml (sample volume) of Tris buffer (1), to the thermostatically controlled cuvette holder at 25 °C, mixed and zeroed at 405 nm. Sample was then added, the solution mixed and the absorbance change per

minute recorded at 405 nm. All samples were assayed simultaneously for trypsin and chymotrypsin in duplicate. Ileal supernatants were assayed directly. Pancreas supernatants were initially activated by preincubation with enterokinase (Glazer & Steer, 1977). Protein determinations were carried out using the modified Lowry procedure (Ohnishi & Barr, 1978). Samples assayed for trypsin were diluted with a BSA/Tris buffer (1 mg/L, pH 8.1) to a protein concentration between 50 -200 µg; typically a 1/100 dilution. Chymotrypsin and trypsin solutions, 0.4 ml and 0.2 ml of each, were pipetted into 1.5 ml Eppendorf centrifuge tubes, mixed with 0.25 ml of enterokinase solution (10 mg/10 ml isotonic saline) and 0.35 ml and 0.55 ml of BSA/Tris buffer respectively. This was incubated at 30 °C for 30 minutes at which time the solutions were assayed directly.

Chymotrypsin and trypsin activities were calculated using a molar absorbtivity of $\varepsilon = 10{,}200$ L mol^{-1} cm^{-1}.

Aminopeptidase N

Aminopeptidase N (Amino-acid arylamidase) in the gastrointestinal mucosa of the jejunal 3 section was assayed on the homogenate supernatant using the principle of hydrolysis of L-alanine-4-*p*-nitroanilide substrate (ANA) with the release of 4-nitoanaline (Hafkienscheid, 1984).

The following solutions were made: (1) Tris buffer (262 mM containing 262 mM NaCl, pH 7.8); (2) Substrate solution ANA (65 mM in methanol) made fresh daily.

Tris buffer (3.1 ml) was preincubated at 30 °C (in the thermostatically controlled cuvette holder), with 0.05 ml of sample for 1min. The substrate solution (0.1 ml) was added, the solution mixed thoroughly and the absorbance change per minute recorded at 405 nm for 5 minutes. Samples were assayed in duplicate.

Units of activity were calculated using a molar absorbtivity of $\varepsilon = 9{,}910$ L mol^{-1} cm^{-1}.

γ-Glutamyl transferase assay

Jugular serum was assayed following a routine method based on the procedure developed by Szasz (1969) which follows the hydrolysis of L-γ-glutamyl-3-carboxy-4-nitroanilide (GLUCANA) substrate with the release of 4-nitoanaline (Shaw, 1984).

The following solutions were made: (1) Tris/glycylglycine buffer (120 mM/120 mM, pH 8.5, containing 0.01 % w/v NaN_3 as a preservative); (2) Substrate solution GLUCANA (35.1 mM, pH 7.5, containing 0.01 % w/v NaN_3).

A working solution was made fresh daily by adding 20 ml of buffer to 2.0 ml of substrate solution. Working solution (2 ml) of this was pipetted into a cuvette with 0.2 ml - x ml sample volume of 0.9 % saline. This was mixed and preincubated at 37 °C (in a thermostatically controlled cuvette holder) for 5 minutes; this stabilized the solution and checked for any spontaneous hydrolysis. Sample (x ml) was then added and the cuvette contents thoroughly mixed. The absorbance change per minute was monitored at 405 nm, for approximately 30 minutes. Samples were assayed in duplicate and if necessary sufficiently diluted so the rate of absorbance change would not exceed 0.2 min^{-1}.

Units of activity were calculated using a molar absorbtivity of $\varepsilon = 9{,}510$ L mol^{-1} cm^{-1}.

Pepsin

Pepsin was assayed in the stomach mucosa and stomach content homogenate supernatants using the principle of haemoglobin hydrolysis to TCA soluble products which were detected at 280 nm (Ryle, 1984).

The following solutions were made: (1) HCl (0.3 mM); (2) TCA (5 % w/v); (3) Neutral haemoglobin solution: 5 g was stirred with 180 ml of water, dialyzed overnight to remove small peptides and made to 200 ml. This was centrifuged, filtered and stored refrigerated; (4) Acidified haemoglobin: Neutral haemoglobin solution was mixed with 0.25 v/v of 0.3 M HCl.

The sample (0.2 ml) was incubated with 1.0 ml of acidified haemoglobin (4) at 37 °C for 10 minutes. TCA (5.0 ml) was then added, the solution well shaken and the sample filtered through Whatman No. 3 filter paper. The absorbance of the filtrate was read at

280 nm against a reagent blank (0.2 ml of saline carried through the above procedure). All samples were assayed in duplicate with an unincubated sample blank with TCA added at the beginning.

Pepsin activity was calculated from the following definition: 1 unit of activity is equivalent to a change in absorbance at 280 nm of 0.001 per minute at pH 2.0, 37 °C.

Serum transaminases

Alanine transaminase (ALT) and aspartate transaminase (AST) were assayed in jugular serum following the methods of Moss *et al.*, 1986; Rej & Horder, 1984; and Horder & Rej, 1984. All procedures use the same principle of coupling the transaminase reaction to a dehydrogenase catalysed reaction and following the oxidation of NADH to NAD^+ at 339 nm.

The following solutions were made: (1) Tris buffer (100 mM, pH 7.6); (2) Pyridoxal-5-phosphate (P-5-P)/Tris (5.44 mM/100 mM, pH 7.6); (3) Substrate α-ketoglutarate/Tris (144 mM/100 mM, pH 7.6): The α-KG was dissolved at 30 °C and the pH adjusted at this temperature and filtered through a 0.22 μM filter; (4) Substrate L-Aspartate/Tris (240 mM/100 mM, pH 7.6): Dissolved and filtered as above; (5) Substrate L-Alanine/Tris (500 mM/100 mM, pH 7.6). Dissolved and filtered as above; (6) Enzyme solution Malate dehydrogenase (MDH)/Lactate dehydrogenase (LDH) (230 kU/L, 105 kU/L in 50 % glycerol); (7) Enzyme solution LDH (420 kU/L in 50 % glycerol).

The following working solutions were made fresh daily and stored at 4 °C. Substrate solutions (20 ml) of L-ASP (4) or L-ALA (5) were mixed with 0.1 ml of enzyme solution MDH/LDH (6) or LDH (7) respectively. Then 0.5 ml of P-5-P (2) was added to both working solutions along with 3.3 mg of NADH, mixed and stored refrigerated.

An aliquot of the working solution (2.0 ml) and 0.2 ml - x ml (sample volume) of Tris buffer was preincubated at 30 °C (in the thermostatically controlled cuvette holder), with x ml of sample, until a stable absorbance at 339 nm was observed. α-KG substrate solution (0.2 ml) was added, the solution thoroughly mixed and the absorbance change per minutes recorded for 30 minutes at 339 nm. Each sample was assayed in duplicate

for both transaminases simultaneously. The sample was initially blanked using a dichromate solution (30 mg/100 ml) diluted 1/10.

Activities were calculated using a molar absorbtivity of ε = 6,300 L mol^{-1} cm^{-1}.

2.2.8 Other standard methods of analysis

Urea and creatinine

Blood and urine were assayed for urea and creatinine on a Technicon auto analyzer. The urea assay was based on the Fearon reaction using diacetylmonoxine. Urea was determined from a standard curve in the range of 0 - 50 g/L for urine and 0 - 20 mM for blood. The creatinine assay was based on the Jaffe reaction using alkaline picrate. Creatinine was determined from a standard curve in the range of 0 - 5 g for urine and 0 - 0.4 mM for blood.

Ninhydrin assay

The assay uses the principle of reacting ninhydrin with free amino groups to form a coloured derivative which absorbs strongly at 570 nm (Hirs, 1967).

Up to 0.2 ml aliquots of the sample were reacted with 0.5 ml of ninhydrin reagent. The tube was shaken incubated in a boiling water bath for exactly 10 minutes, cooled in cold water for 10 minutes after which 2.5 ml of 50 % ethanol was added. The tubes were vortexed and the absorbances read at 570 nm against a blank. Each run consisted of duplicate blanks containing water instead of sample and a duplicate standard of 0.05 ml leucine (1.5 mM).

Protein estimation (modified Lowry)

The principles of both the Biuret (peptide bond) and Lowry (reduction of Folin-Ciocalteu phenol reagent by tyrosine and tryptophan) methods were used in this assay to form a sensitive complex which absorbs strongly at 700 nm (Ohnishi & Barr, 1978).

Up to 1 ml of sample was mixed with 4.0 ml of a diluted Biuret (DB) reagent (1 vol biuret + 7 vol 2.3 % Na_2CO_3, made fresh daily) and left to stand for 10 minutes at room temperature. Folin-phenol reagent (0.125 ml) was then added and the solution mixed and left to stand for a further 30 minutes. The absorbance was read at 700 nm against a

blank. A standard curve was constructed in the range of 0 - 500 mg using a 5 g/L BSA solution.

Dry matter determinations

Samples were weighed into small pyrex beakers which had been dried in an oven at 105 ^{o}C for 15 minutes and cooled in desiccators over silica gel. Samples were dried at 105 ^{o}C for 16 hours, cooled in desiccators over silica gel and weighed.

Urine analysis tests

Specific gravity - using a pycnometer.

Osmolality - Advanced Digimatic Osmometer, Model 3D2 (Advanced Instruments Inc, Massachuetts, USA).

Clinistick dipstick tests - Multistix 2820 A Ames dipstick tests for pH, protein, glucose, ketones (AAA), bilirubin, blood and urobilinogen.

Erhlich's test for hexosamines (Henry, 1964) - Urine (1 ml) was added to 1 ml of acetoacetone solution (1 ml acetoacetone plus 50 ml of 0.5 N $NaCO_3$). Water (1 ml) was added to this and the solution heated for 15 minutes in a boiling water bath. Once cool 5 ml of ethanol was added and the mixture shaken. To this 1 ml of Ehrlich's reagent (0.8 g of *p*-dimethylaminobenzaldehyde reagent plus 30 ml of alcohol plus 30 ml of concentrated HCl) was added along with 1 ml of ethanol. The solution was thoroughly mixed and left to stand for 30 minutes. A violet colour was a positive indication of the presence of hexosamines.

Fluorescence test for porphyrins (Bradley & Benson, 1974) - Urine (10 ml), in a test tube, was irradiated in dark with UV light. A red-orange fluorescence indicated the positive presence of uroporphyrins.

Indicans test (Bradley & Benson, 1974) - To 5 ml of urine in a test tube 5 ml of Ferric chloride reagent (0.2 % in conc HCl) was added and the solution mixed. Chloroform (2 ml) was then added the sample settled and observe. When indican was present the chloroform layer showed a deep violet or blue colour for a positive result. Normal urine may give a faint blue colour.

Alkaptoneuria (Bradley & Benson, 1974) - To 2 ml of urine in test tube, 10 % NaOH was added drop by drop. The urine will darken in alkaline solution if positive. To 2 ml of urine in test tube, 10 % $FeCl_3$ was added drop by drop. A transient blue colour was seen if positive.

Iron content (Jacob, 1986) - Urine (10 ml) was freeze-dried to a powder. The powder was ashed in a furnace at 500 °C for several hours. The ash was taken up in 2.5 ml of 2 M HCl. The supernatant was loaded onto an Atomic Absorption Spectrophotometer (Instrumentation Laboratory, aa/ae spectrophotometry 457) for the determination of the iron content. Values were expressed in parts per million (ppm)/ml of urine.

Absorbance spectrophotometry - Urine was diluted 1/25 with water and scanned between 200 and 600 nm on an HP 8452A spectrophotometer.

Chapter 3

THE PRODUCTION AND ANALYSIS OF EARLY MAILLARD BROWNED CASEIN

This Chapter concerns the heat treatment of a model casein-glucose mixture to produce an early Maillard browned casein. In addition, the analysis of the proportions of early and advanced Maillard products present in this mixture is described.

3.1 Introduction

The effects of heat on a variety of protein sources have been investigated, mainly in relation to their use in the food processing industry. Examples of such protein sources are milk products, soy bean meals, egg products, fish meals, cereals and meat and bone meals. However, because of the complexity of the Maillard reactions it is useful to use model systems of pure proteins and reducing sugars, to simplify the chemistry. The most extensively studied model systems have been those involving: bovine milk proteins, especially casein and its interactions with glucose (Smith & Friedman, 1984; Moller *et al.*, 1977 I, II & III; Knipfel *et al.*, 1975; Dworschak & Hegedus, 1974; Clark & Tannenbaum, 1970 & 1973; Rao & McLaughlan, 1967; and Lea & Hannan 1949 I, 1950 II, 1950 III &1951 IV); and egg albumin and its interaction with glucose (Tanaka *et al.*, 1975 A; Tanaka *et al.*, 1977; Kimiagar *et al.*, 1980; and Pintauro *et al.*, 1983). These proteins have a high protein quality containing most, if not all, of the essential amino acids in relatively high concentrations. Consequently, they can be used as the sole protein source in experimental diets. However, although browned egg albumin has been extensively used, in studying the physiological aspects of the ingested Maillard products, browned casein has been poorly investigated.

Casein is the major group of proteins in milk, constituting 80 % of the total protein content. Cows milk is important in relation to human diets, particularly for babies who are often dependant on this source of intake as the single source of nourishment and,

furthermore, in milk concentrates and milk biscuits supplied to under-developed countries. For these reasons, casein was used in this work.

Several methods of heat treatment have been used in nutritional studies, most of which mimic the types of heat processing undertaken in the food industry, e.g. storage temperatures; drying processes (force-draft oven, roller-drying and spray-drying); sterilization processes (autoclave); and flavour production, by baking and roasting. The autoclave was chosen in this study as it is the simplest and most rapid method for producing variable intensities of heat treatment.

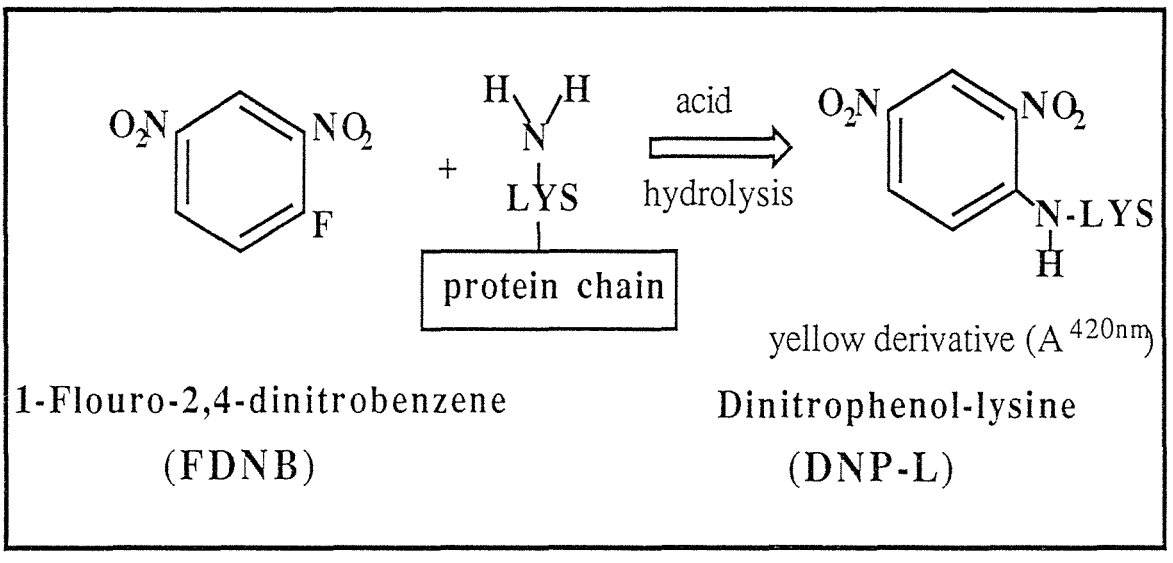
Previous studies have examined the effects of extensive Maillard reactions. Very severe heat damage may result not only in advanced Maillard products and extensive destruction of lysine, but also the destruction of other heat labile amino acids and the formation of protein-protein interactions through isopeptide cross linkages (Mauron, 1972; and Friedman, 1977). Early Maillard damage, if it results in extensive lysine losses, in physiological experiments, may result in nutritional inadequacies if it is not supplemented for. In relation to human nutrition, the heated foods which are consumed probably contain about equal propotions of early and advanced Maillard products.

The extent of the Maillard reaction has been investigated using several techniques. In particular, lysine and its availability has been extensively investigated, since the first consequence of the Maillard reaction in protein sources is the loss of the lysine normally available to an animal. As lysine is one of the essential amino acids, its estimation and nutritional significance are extremely important for quality control in the food and feedstuffs industry. In summary (Carpenter & Booth, 1973), Block and Mitchell (1946) reviewed the correlation of the amino acid composition of proteins, with their respective nutritive values, and reported that amino acid analysis was adequate for predicting the nutritive value of many food sources. However, it became recognized that some materials, such as milk products which were heat processed or stored under adverse conditions, gave a lower nutritive value than that expected from the corresponding amino acid analysis. The "paradox" of lysine detected as being present by amino acid analysis but nutritionally unavailable, attracted considerable interest. Subsequent studies involving the mild heat treatment of milk powders and casein-glucose model systems (which produced only the early Maillard reaction) showed that the reaction between carbonyl groups of the reducing sugars and free amino groups formed compounds which were

nutritionally unavailable, but could be acid hydrolysed to free lysine. Consequently, several *in-vitro* methods were developed to assess nutritionally available lysine. These included biological assays, enzyme and microbial methods and chemical methods. Assessment of the accuracy of these techniques has been recently reviewed by Hurrell and Carpenter (1981). They concluded that the best methods for lysine determination in the presence of early Maillard products (which effects lysine estimations because of its similar basic reactive nature) are the direct 1-fluoro-2,4-dinitrobenzene (FDNB) reactive lysine method, total lysine after borohydride treatment, guanidation and, in the specific case of milk powders, the furosine technique. A later study, specifically on the storage of milk powders under adverse conditions, confirmed the observation that: (1) direct FDNB reactive lysine ; and (2) reactive lysine from furosine, were the most reliable methods (Hurrell *et al.*, 1983):

(1) The FDNB reactive lysine procedure of Carpenter (1960) as modified by Booth (1971) is probably the most widely used technique in the assessment of nutritionally available lysine. FDNB was the first reagent used for measuring reactive lysine in foodstuffs. The FDNB combines with free amino groups, but the technique only detects the lysine with a free ε-amino terminal exposed - other reactants have been extracted (i.e. N-terminal and free amino acids, arginine and histidine). The yellow derivative is estimated spectrophotometrically at 420 nm (Figure 3.1).

Figure 3.1 *The FDNB available lysine reaction.*



(2) The furosine technique is not only useful in assessing nutritionally available lysine, but also in the detection of Amadori compounds (ketosyl-lysines, such as lactulosyl-lysine and fructosyl-lysine) from their acid hydrolysis products pyridosine, regenerated lysine and furosine (Figure 3.2). These are measured by the ion-exchange chromatography procedure used for amino acid analysis. Lactulosyl-lysine (ε-DL-L) is formed from the reaction of lactose with lysine and fructosyl-lysine (ε-DF-L) is formed from the reaction of glucose with lysine. Both yield the same products under the same acid hydrolysis conditions in the same proportions (Moller, 1981). The historical background to the identification of these compounds are discussed by Finot *et al.* (1981). In summary, the structures of furosine and pyridosine were identified and named by Finot *et al.* (1968 & 1969 respectively). Subsequent synthesis and analysis of free and protein bound lactulosyl-lysine enabled (from the furosine formed and total lysine content, after acid hydrolysis) the determination (using a simple set of ratios and equations, presented in the methods section of this Chapter, 3.2.3 - developed by Bujard & Finot, 1978) of: lysine as lactulosyl-lysine; nutritionally available lysine (reactive lysine); and the percentage of lysine blocked in the form of Maillard products . However, since there is some evidence (Finot & Mauron, 1972) to suggest that the proportions of acid hydrolysis products (pyridosine, regenerated lysine and furosine) may vary under different acid hydrolysis conditions, it is necessary to synthesize free ε-DF-L and determine its behaviour under the acid hydrolysis conditions used.

From the above equations and estimation of the lactulosyl-lysine and reactive lysine (discounting the regenerated lysine from the hydrolysis of lactulosyl-lysine) contents, it is possible to calculate the relative proportions of early and advanced Maillard products in relation to the lysine lost. This method has previously been used on milk powders heated for several weeks at 70 ^{o} C (Hurrell & Carpenter, 1981), Figure 3.3. Initially, as lactose reacts proportionately with lysine the reactive lysine decreases and lactulosyl-lysine increases, as this obeys a stoichiometric reaction. In subsequent reactions lactulosyl-lysine is converted into advanced Maillard products and the content of lactulosyl-lysine decreases. Therefore, simply by determining the difference between the reactive lysine content in the non-heated sample and the lactulosyl-lysine and reactive lysine contents in the heated sample, lysine in the form of advanced Maillard products (lysine destroyed) can be estimated.

Figure 3.2 *Acid hydrolysis products of the Amadori compounds ε-deoxyfructosyl-lysine and ε-deoxylactulosyl-lysine.*

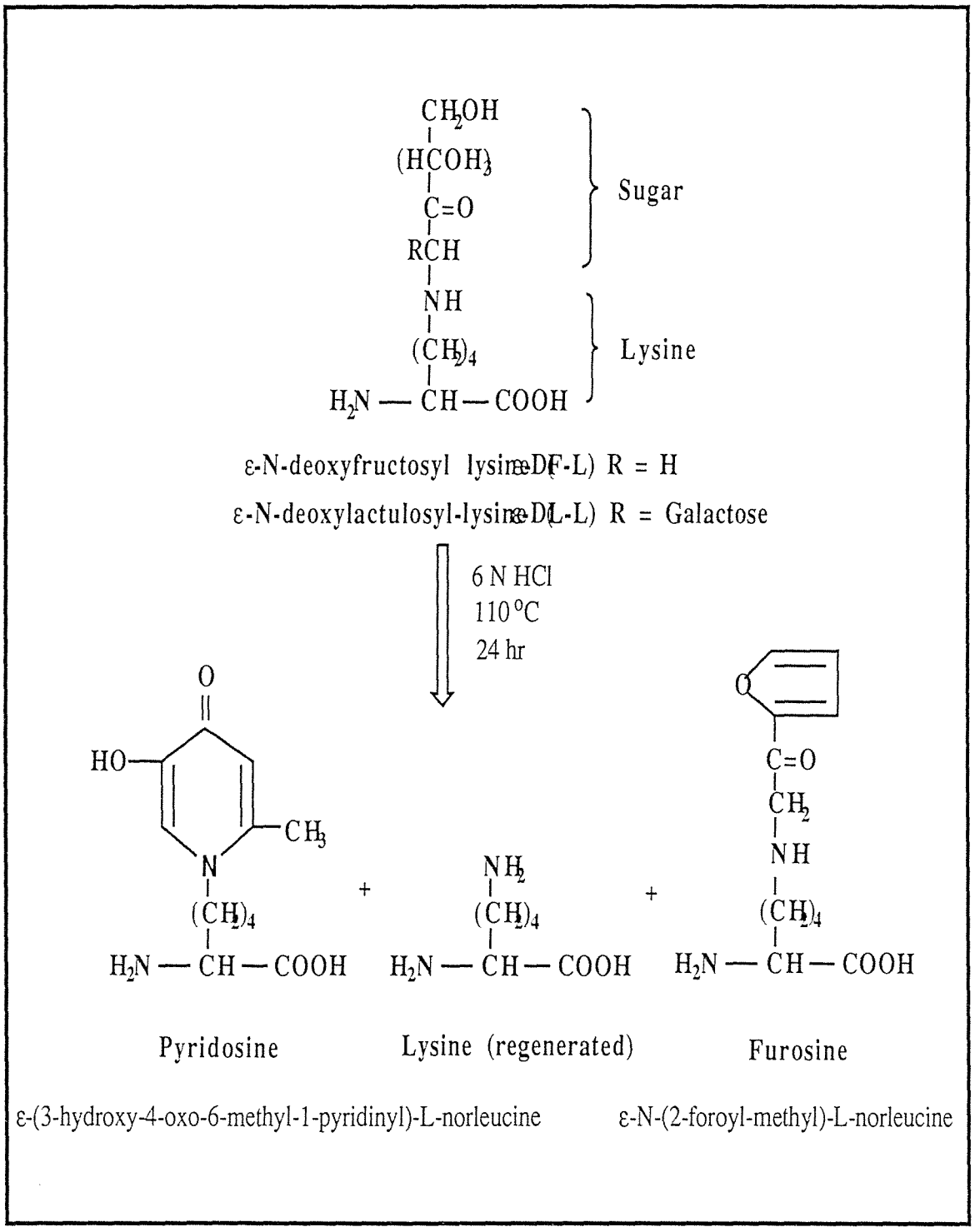
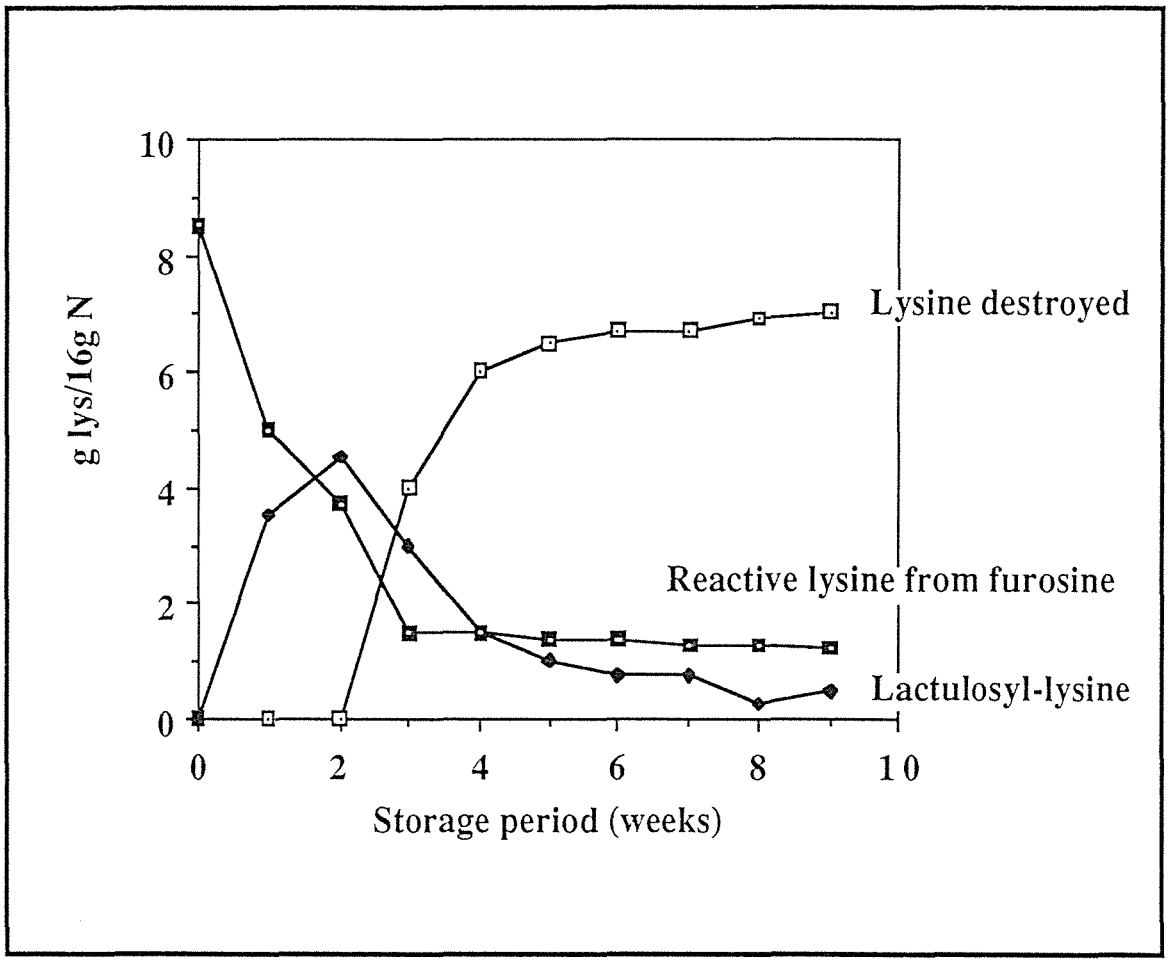


Figure 3.3 *The distinction between lysine present as early and advanced Maillard products in milk powders heated for several weeks at 70 °C (Hurrell & Carpenter, 1981).*



Although lysine is the most extensively damaged amino acid during the Maillard reaction, it has been documented that other amino acids may also be affected by the heat treatment used. These include arginine and histidine (Lea & Hannan, 1950 III; and Tanaka *et al.*, 1975 A), cystine (Miller *et al.*, 1965), methionine (Rao & McLaughlan, 1967), tryptophan (Tanaka *et al.*, 1975 A; and Nielsen *et al.*, 1985) and tyrosine (Lea & Hannan, 1950 III). However, in the specific case of mild heat treatment to casein and glucose only the basic amino acids were reported (Lea & Hannan, 1950 III) to be lost.

A further estimate of the extent of the Maillard reaction is from the loss of the reducing sugars. Its interaction in the Maillard reaction can be easily determined by an estimation of its free concentrations before and after heat treatment. Lea and Hannan (1950 II) have studied the interactions of glucose with casein and demonstrated its usefulness in

distinguishing the amount of glucose reacted as early and advanced Maillard products calculated from the lysine lost and concentrations of fructosyl-lysines formed. In addition, $U^{14}C$-glucose has been used to label Maillard compounds and is a useful analytical tool for the identification of Maillard products both *in-vitro* and *in-vivo* (Erbersdobler, 1977; and Finot & Magnenat, 1981).

The overall objective of this study in the present Chapter was to produce an early Maillard browned casein with relatively high proportions of Maillard products from the reaction of glucose and lysine, but with minimal damage to other amino acids. Furthermore, radioactive U-^{14}C-glucose was used to label the Maillard compounds. The specific aims were:

(1) - To synthesize ε-DF-L and determine its hydrolysis behaviour under the acid hydrolysis conditions used in the present research, for the estimation of its concentrations in the heated samples.

(2) - To characterize the extent of the Maillard reaction in the early Maillard browned casein, by evaluating the proportions of early Maillard products (ε-DF-L) and advanced Maillard products in relation to the lysine and glucose lost.

3.2 Experimental

The standard chemical methods of analysis are reported in detail in Chapter 2: The amino acid composition of the casein-glucose mixtures were determined by acid hydrolysis (6 M HCl containing 0.1 % phenol) in an evacuated sealed tube (2.2.3). Lysine was also determined by the direct FDNB reactive lysine procedure (2.2.1) of Carpenter (1960) modified by Booth (1971). Glucose was determined by the dinitrosalicylic acid and the direct hexokinase methods (2.2.2). Total nitrogen (2.2.4) was determined by an automated Kjeldahl technique (A.O.A.C., 1975).

3.2.1 Synthesis and acid hydrolysis of the Amadori compound, ε-deoxyfructosyl-lysine (ε-DF-L)

The ε-DF-L was synthesized, by reacting α-N protected lysine with glucose, and purified according to the method devised by Dr M. Lever, Christchurch Hospital, Christchurch (personal communication)[1]. The purified white powder was dissolved in water (approximately 3 mg/ml) and 25 µl loaded onto a Beckman 120 C amino acid analyzer, to determine the area under the chromatogram. Dry matter determinations were also carried out on the solution to determine the concentration of unhydrolyzed ε-DF-L loaded.

A portion of the above solution of ε-DF-L (100 µl), was freeze-dried and subjected to one of two types of acid hydrolysis: (1) either in 1 ml of 6 M HCl in an evacuated sealed tube for 24 hours at 110 °C; or (2) under the same conditions, but with the inclusion of 0.1 % phenol in the acid hydrolysis mixture. The hydrolysates were dried, dissolved in a volume of 1 ml of buffer and 250 µl quantitatively loaded (this was equivalent to 25 µl of hydrolysed ε-DF-L) to determine the areas of the eluted peaks under the chromatograms.

3.2.2 Production of the non-heated and heated casein-glucose mixtures

Initially, an attempt was made to prepare an early Maillard browned protein by the procedures of Smith and Friedman (1984) and Lea and Hannan (1949 I), in which various proportions of casein and glucose were dry-blended. These mixtures were autoclaved at 121 °C for up to 15 min. The colours of the products progressively intensified to a very dark brown. Analysis of the heated mixtures based on the methods of Smith and Friedman (1984), showed that the high concentrations of glucose caused excessive caramelization, resulting in unmanageable material, whereas heated mixtures based on the methods of Lea and Hannan (1949 I), resulted in a maximum amount of lysine loss of 35 %, in spite of intense browning. Consequently, in the latter procedure casein and glucose were blended together in solution and the mixture dried before autoclaving. This improved the interaction between casein and glucose, and autoclaving for only one to two minutes resulted in the formation of more Maillard products than was

[1] The methodology of this synthesis and purification is withheld on the grounds of a disclosure agreement for patentcy.

possible with dry-blending. This procedure was, therefore, used to produce an early Maillard browned protein.

Bovine casein and anhydrous glucose were combined in all mixtures prepared, to provide final solids of 88 % casein (C) and 12 % glucose (G). Preliminary work was carried out on in the laboratory on the solution blending procedure. Casein was suspended in water and adjusted to pH 7.0 using 1 M NaOH. The solution was heated to 50 °C to aid dissolution. Glucose was then added and the mixture stirred for 30 minutes at 50 °C, before being transferred to a round bottomed flask and freeze-dried to a white crystalline powder. The freeze-dried powder was heated in an autoclave to varying intensities of browning to determine the extent of lysine and glucose losses in relation to the brown pigment formation.

To prepare the final casein-glucose mixtures all operations had to be scaled up for the large quantities of material needed. Equipment from the Dairy Research Institute (Palmerston North) was used for blending and spray-drying. A diagrammatic summary of these procedures is given in Figure 3.4. Two casein-glucose mixtures were prepared, one for the non-heated mixture and the other for the heated mixture.

Non-heated mixture (A)

Lactic acid precipitated curd was added to an equal volume of water (20 % solids), homogenized and pumped to a solution-mixer heated by a water jacket. The homogenate was heated to 70 °C and slowly adjusted to pH 7.0 (using a solution of 2.2 % NaOH; 495 g NaOH/100 kg suspension) to suspend the precipitated curd. The hot sodium-casein suspension was pumped into a spray-drier holding tank, stirred further and the temperature adjusted to 55 °C. Once the suspension had stabilized at this temperature, it was pumped into a spray-drier where it was atomized at the top and dried by snap heating from hot convection currents forced from a large fan at the bottom. The dry powder was collected (40 °C) at an outlet port at the bottom of the tank and cooled. As spray-drying involved heating the spray-dried sodium caseinate (22 kg) was dry-blended with anhydrous glucose (3 kg) in the spiral-paddle dry-mixer for several hours. The dry mix was stored in a large plastic lined bag in a cold room (5 °C). This was designated the non-heated casein-glucose mixture (A).

Heated mixture (B)

The same procedure as detailed above was used, except that the glucose was added as a slurry (12 % of the solids) to the solution-mixer and the suspension stirred at 70 °C for a further 15 minutes before spray-drying. The above procedure was carried out in three 100 kg (20 kg total solids) batches. The different batches were then dry blended in the spiral-paddle dry-mixer.

Portions (2 kg) of the blended material were spread evenly over 4 trays at a sample depth of 0.7 cm. Each tray was covered with an aluminium foil lid, stacked in the middle of an autoclave, and heated at 121 °C for 1 minute. The resultant browned material was crushed, passed through a 1 mm mesh sieve and all the portions dry-blended in the spiral-paddle dry-mixer.

Additional heating of this material was necessary as there was less than the expected 50 % lysine loss. This was carried out in a force-draft drying oven. Four large trays each holding 5 kg and a fifth containing water (to maintain a moist atmosphere) were heated at 70 °C for 1 day and then dry blended in the large spiral-paddle dry-mixer. The resultant dry mix was stored in large plastic lined bags in a cold room (5 °C). This was designated the heated casein-glucose mixture (B).

Radioactively labelled heated mixture (BR)

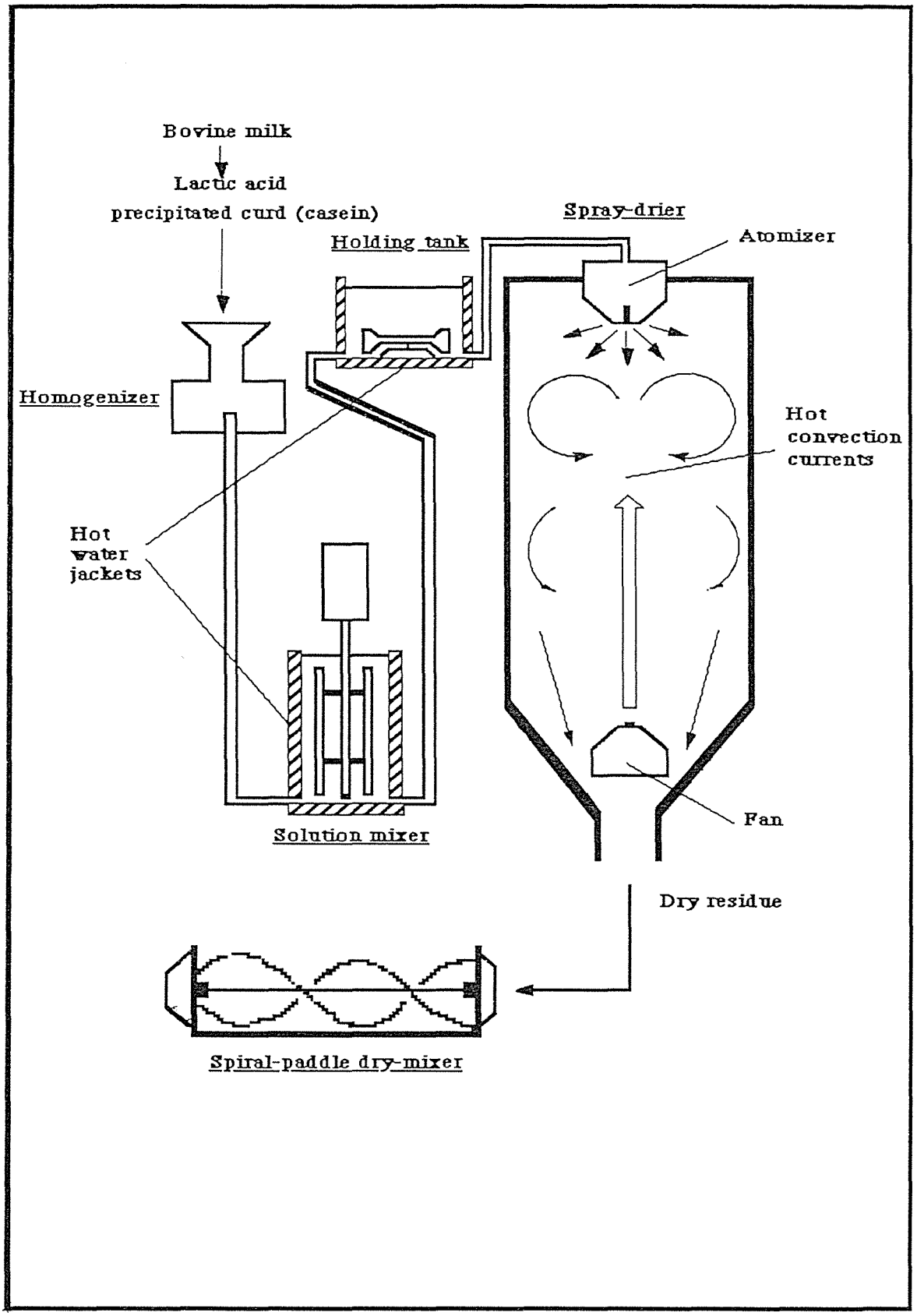
The production of this mixture was modeled on the procedure used for the production of B, but laboratory equipment was used. Care was taken to minimize radioactivity contamination. Sodium caseinate (880 g) was suspended to 4.4 litres of water, the temperature raised to 70 °C and the suspension adjusted to pH 7.0 (using 10 M NaOH). This operation was carried out in a large stainless steel canister heated by a water bath while the mixture was constantly stirred with a paddle stirrer. When a consistent, creamy, viscous syrup had formed, 120 g of anhydrous glucose was added as a slurry (12 % of the solids). To this, 1 mCi of radioactive D-(U-^{14}C)-glucose was added and the mixture stirred at 70 °C for a further 15 minutes. While still hot, the mixture was poured into large (water-tight) stainless steel freeze-drying trays and freeze-dried. This freeze-dried powder was crushed and passed through a 1 mm mesh sieve.

The powder was spread evenly over a large tray at a sample depth of 0.7 cm and autoclaved to a colour matching that of the heated mixture (B). The autoclaved mixture

was resuspended in 5 L of water at room temperature and the mixture adjusted to pH 7.0 (using 10 M NaOH). The unreacted glucose was then removed by precipitating the protein at pH 4.5 and filtering the precipitate using muslin cloth. The precipitate was resuspended and the process repeated four times. The mixture was not heated. The final precipitate was resuspended at 70 °C and the mixture adjusted to pH 7.0 (using 10 M NaOH). The suspension was freeze-dried, crushed, passed through a 1 mm mesh sieve and dry-blended. The dry mix was stored in a large plastic lined bag in a cold room (5 °C). This was designated the radioactively labelled heated casein-glucose mixture (BR).

Figure 3.4 Processing of sodium-caseinate commonly follows this procedure, from lactic acid precipitated curd to the final spray-dried powder. In the case of the non-heated mixture (A) the glucose was added last in the dry-mixer. In the case of the mixture for the production of the heated mixture (B), glucose was added to the suspended casein in the solution-mixer, mixed and spray-dried to a powder. All processes were carried out at the Dairy Research Institute (DRI), Palmerston North, New Zealand.

Figure 3.4 *The blending procedures used for the production of the non-heated (A), and heated (B) casein-glucose mixtures.*



3.2.3 Analysis of the non-heated and heated casein-glucose mixtures

The Amadori compound, ε-deoxy-fructosyl-lysine (ε-DF-L), was identified from the detection of its acid hydrolysis product, furosine, and estimates from the response factors calculated from the hydrolysis of the synthesized and purified ε-DF-L (see Results 3.2.1).

The values determined on the heated casein-glucose mixtures (B & BR) were compared with a set of formulas developed by Bujard and Finot (1978). These determine the percentage of blocked lysine from the total lysine and furosine content in milk powders and are based on lysine response factors (Finot *et al.*, 1981).

Lysine as lactulosyl-lysine (LL)	=	3.1 fur.
Regenerated lysine (RL)	=	40 % LL = 1.24 fur.
Nonregenerated lysine (NRL)	=	60 % LL = 1.86 fur.
Total lysine (TL)	=	Reactive lysine + RL.
Initial lysine (IL)	=	TL + NRL
Reactive lysine	=	TL - RL.
% blocked lysine	=	$\frac{LL}{IL} \times 100 = \frac{3.1\ fur}{TL + 1.86\ fur} \times 100$

The percentage of blocked lysine does not apply in the present study as this is for the specific case of the early Maillard reaction and does not account for the lysine blocked in the form of advanced Maillard products.

Distinction of the lysine and glucose lost into early and advanced Maillard products

The total moles of lysine lost in the heated mixtures (B & BR) was determined by the comparison with the non-heated mixture (A) of the 1-fluoro-2,4-dinitrobenzene (FDNB) reactive lysine estimates. The total moles of glucose lost was estimated by comparison with the glucose content of the control non-heated mixture (A). Since a stoichiometric reaction holds for the condensation of lysine with glucose to form one molecule of ε-DF-L, the calculated moles of ε-DF-L are equivalent to the moles of lysine or glucose that have combined. If it is assumed that all of the early Maillard products are in the form of ε-DF-L, the percentage of lysine and glucose lost can be calculated as early Maillard

products. The remaining percentage was assumed to be that attributed to the lysine and glucose lost as advanced Maillard products.

The incorporation of U-^{14}C-radioactive glucose into Maillard products was used as a labelling method for the distinction into early and advanced Maillard products. From the amount of ε-DF-L present, and the dilution factor of radioactive glucose with cold glucose, the radioactivity attributed to ε-DF-L was calculated (dpm/mol). From the total radioactivity and the total amount of ε-DF-L in heated samples, the radioactivity by difference was considered as being due to glucose bound or reacted in the form of advanced Maillard products, this was designated residual radioactivity.

3.3 Results

3.3.1 Determination of the Amadori compound ε-deoxy-fructosyl-lysine (ε-DF-L)

The synthesized and purified ε-DF-L was a very hygroscopic, white, crystalline powder. The ion-exchange chromatogram of the unhydrolyzed ε-DF-L (Figure 3.5 I) identified one major ninhydrin positive peak with a retention time (RT) = 66 min. Two other relatively smaller peaks were also noted. Acid hydrolysis of ε-DF-L under conditions without phenol (6 M HCl; 110 °C; 24 hr) and with phenol (6 M HCl containing 0.1 % phenol; 110 °C; 24 hr), produced three smaller peaks which corresponded to pyridosine RT = 64 min, lysine RT = 87 min and furosine RT = 152 min (Figure 3.5 II & III respectively). An unidentified peak (RT = 44 min) also eluted from the hydrolysate without phenol. The peak areas of ε-DF-L before hydrolysis, along with the areas of the products formed after hydrolysis (measured by an integrator) and also expressed as a percentage of the peak area of ε-DF-L are reported in Table 3.1. The percentages obtained by Moller (1977 II) are also included in Table 3.1.

Under acid hydrolysis conditions without phenol, the pyridosine and lysine percentage areas (of ε-DF-L) were similar to those obtained by Moller (1977 II), but the furosine recovered was approximately double. Under acid hydrolysis conditions with phenol, however, pyridosine and furosine were similar to those from Moller (1977 II), but the

recovery of lysine was approximately 1.5 times more than that reported. The inclusion of phenol in the acid hydrolysis mixture thus caused an increase in the pyridosine area (1.33 times) and the lysine area (1.62 times), but decreased the furosine area to decrease (0.54 times), compared with the acid hydrolysis of ε-DF-L without phenol. As all acid hydrolysis in the present study involved the use of 0.1 % phenol in the acid hydrolysis mixture, the proportions of the products formed under these conditions were used in the following calculations.

From the dry matter determination, the concentration of the ε-DF-L was 2.57 mg/ml. Using the molecular weight of ε-DF-L (308), the amount loaded equates to 209 nmol/25 µl. The corresponding furosine integrator area from the phenol-acid hydrolysis of this was 6,565,473. Therefore, the furosine area per nmol of ε-DF-L equates to:

(1) Area of furosine / nmol of free ε-DF-L = $\frac{6,565,473}{209}$ = 31,414 area/nmol.

However, Finot *et al.* (1981) reported that the hydrolysis of protein bound lactulosyl-lysine yields 32 % furosine, and not the 20 % furosine as for the free compound. This was also assumed to be the case for fructosyl-lysine (ε-DF-L, since acid hydrolysis of chemically synthesized free lactulosyl-lysine, fructosyl-lysine, acetyl-lactulosyl-lysine and formyl-lactulosyl-lysine, and separation of the hydrolysis products by ion-exchange chromatography yield the same area ratios (Moller, 1977 II). Therefore, the area of furosine/nmol of protein bound ε-DF-L will be higher by a factor of 1.6 (32 % / 20 %):

(2) Area of furosine / nmol of bound ε-DF-L = 31,414 x 1.6 = 50,262 area/nmol.

Furthermore, Moller (1981) has reported that when ε-DF-L was protein bound, the proportion of lysine released under acid hydrolysis conditions also changed, from 50 % (for the free molecule) to 40 % of the ε-DF-L area, i.e. from 2.50 x furosine area to 1.25 x furosine area (a factor of 0.5 different). This again has been assumed to occur under the acid hydrolysis conditions used in the present study. Therefore, the area of lysine from the hydrolysis of protein bound ε-DF-L equates to 1.6 x furosine area compared to 3.2 x furosine area for the free molecule, i.e. by a factor of 0.5.

In calculating the nmoles of ε-DF-L from the above "response factors" (integrator area of furosine per nmol of ε-DF-L), two additional problems were considered. The first was the response factor fluctuations between runs, due to differences in the colour development with ninhydrin. This was counteracted by normalizing all of the lysine response factors (integrator area per nmol of lysine), from the various runs, to the lysine response factor used in the above calculations of 162,166 area/nmol of lysine (this was carried out instead of loading the hydrolysed ε-DF-L on for each run, as there was insufficient to do this). The second consideration was that in cases where the area of furosine was too small for the integrator to measure accurately, chart recordings were run at the most sensitive scale and the area measured manually.

Figure 3.5 Ion-exchange chromatography was carried out on a Beckman 119BL amino acid analyser, using a three buffer system (refer Chapter 2, 2.2.3). The corresponding area values (measured using an integrator) under the chromatograms are given in Table 3.1.

Figure 3.5 *Ion-exchange chromatograms of (I) ε-DF-L; and the products formed by acid hydrolysis of ε-DF-L with (II) 6M HCl or (III) 6 M HCl containing 0.1 % phenol.*

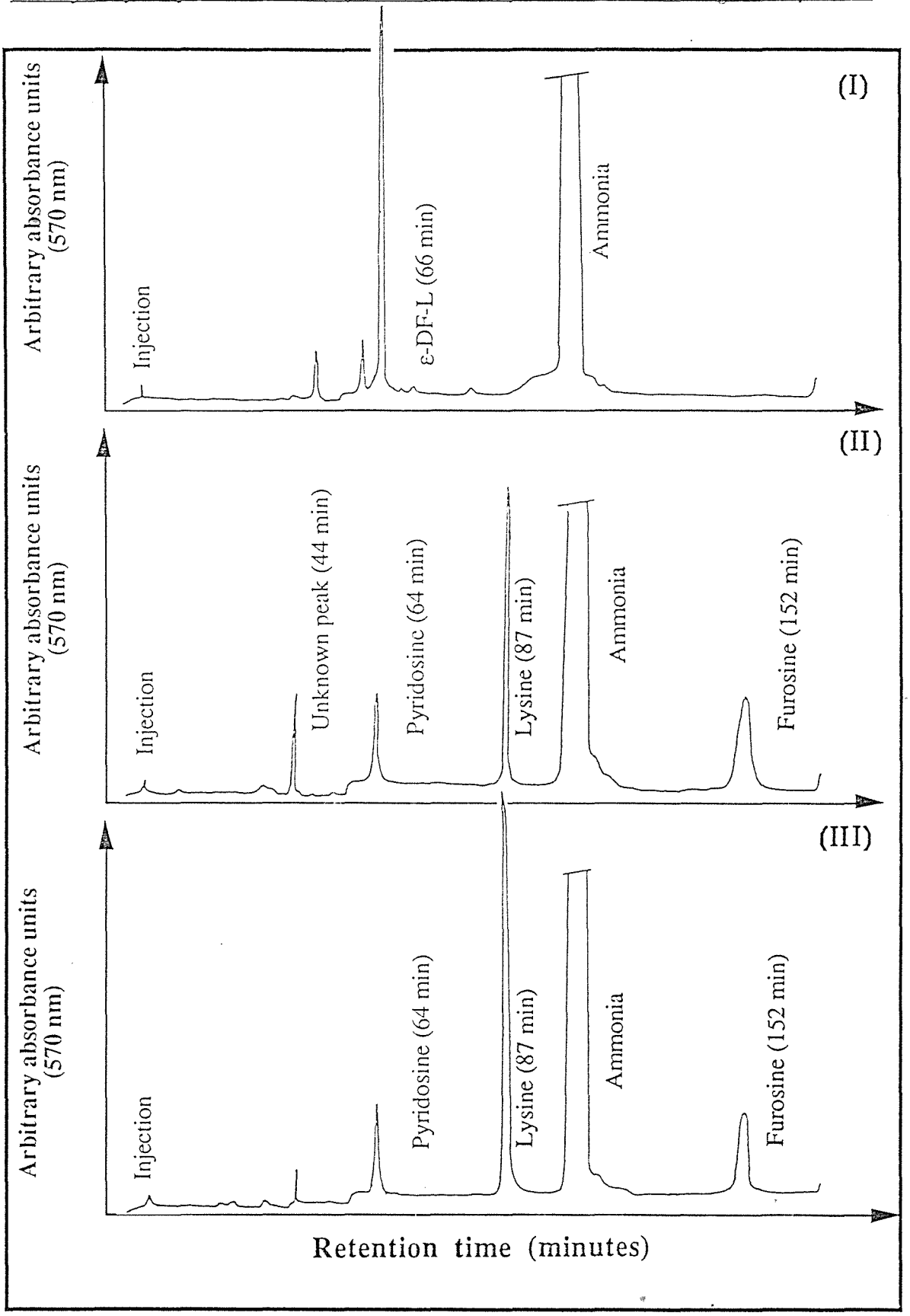


Table 3.1 These area values are taken from the chromatograms reported in Figure 3.5. Values in parentheses are from Moller *et al.* (1977), using fructosyl-lysine analysed before and after acid hydrolysis (6 M HCl, 112 °C, 24 hr under reflux).

Table 3.1 *Integrator areas of (I) ε-DF-L; and the products formed by acid hydrolysis of ε-DF-L with (II) 6M HCl or (III) 6 M HCl containing 0.1 % phenol. The ratios of the integrator areas of the products to each other or as a % of the ε-DF-L integrator area are included together with the comparable values of Moller (1977 I)*

Sample	Area	% of ε-DF-L area	Ratios
(I) ε-DF-L	28,754,433	100 (100)	
(II) 6 M HCl; 110 ºC; 24 hr			
Pyridosine (P)	2,592,753	9.0 (10)	P/L = 0.200 (0.2)
Lysine (L)	12,980,683	45.1 (50)	F/L = 0.933 (0.4)
Furosine (F)	12,105,063	42.1 (20)	P/F = 0.214 (0.5)
(III) 6 M HCl; 0.1 % phenol; 110 ºC; 24 hr			
Pyridosine	3,437,488	12.0 (10)	P/L = 0.164
Lysine	21,016,149	73.1 (50)	F/L = 0.312
Furosine	6,565,490	22.8 (20)	P/F = 0.526

3.3.2 Production of the casein-glucose mixtures

In the preliminary work, a blended solution of casein and glucose was freeze-dried and subjected to varying degrees of heat treatment to produce yellow to dark brown mixtures. These were subsequently analysed for FDNB reactive lysine and glucose content to estimate the extent of the Maillard reaction. The colours of the various samples and the relationship between the FDNB reactive lysine and glucose losses are graphed in Figure 3.6, and compared with the stoichiometric line for a molar glucose-lysine reaction. This shows that progressively more glucose than FDNB reactive lysine was lost as the Maillard reaction intensified in brown pigment production. Even so, the results indicated the production of much more early Maillard products than was obtained by dry-blending (refer methods 3.2.2).

In the large scale production of the casein-glucose mixture 120 L were spray dried, as freeze-drying was impractical. This resulted in a high loss (40 %) by weight through material adhering to the inside of the spray-drying tank. Analysis of the spray-dried powder showed that approximately 10 % of the total FDNB reactive lysine and 15 % of

the total glucose had been lost, this material was also slightly off white in colour. Assuming that all the Maillard products were in the early stage in this mixture, the glucose and lysine molar losses should be approximately equivalent, i.e. for an equimolar reaction 10 % of the total lysine would have combined with 8 % of the total glucose, by weight. Therefore, an extra 7 % of the glucose (in the mixture) had been lost due to spray-drying, other than its involvement in the Maillard reaction.

Tests were carried out on a small batch of the spray-dried material, to establish the autoclave conditions which would give the appropriate colour for a 50 % lysine loss and a 65 % glucose loss. This was achieved by autoclaving the mixture at 121 °C for 1 minute. However, when the material was autoclaved in large batches (2 kg), it did not mimic the small batch experiments, with the mixture only losing approximately 30 % of the total lysine and 45 % of the total glucose. A gradation of browning was found in the trays around the edges where steam had jetted in, as well as due to a temperature gradients inside the autoclave chamber, with the centre of the chamber being the least brown. Therefore, it was necessary to further heat the samples in a force-draft oven to the appropriate colour.

In view of the above complications, autoclaving of the radioactive casein-glucose mixture for the production of (BR) was carried out in smaller batches of only 250 g per tray, one tray at a time to a colour matching that of the non-radioactive heated casein-glucose mixture (B).

3.3.3 Analysis of the casein-glucose mixtures

The amino acid composition of the non-heated (A), heated (B) and radioactively labelled heated (BR) casein-glucose mixture (Table 3.2) show that no other amino acids in the heated mixtures (B and BR) have been lost apart from lysine, arginine and to a very small extent histidine.

The total lysine content, total glucose content, proportions of early and advanced Maillard products (expressed as a proportion of the total lysine lost) in the casein-glucose mixtures (A, B and BR) are reported in Table 3.3.

Since the radioactively heated mixture (BR) was freeze-dried and not spray-dried, glucose was not lost other than its involvement in the Maillard reaction, whereas, comparatively for similar FDNB reactive lysine losses, the heated mixture (B) had lost an extra 7 % glucose due to spray-drying. However, comparison of the heated mixtures showed similarity in the extent of the Maillard reaction. Approximately 50 % of the FDNB reactive total lysine and 70 % of the total glucose were lost, almost double the glucose to lysine loss on a molar basis. The proportions of early and advanced Maillard products (expressed as a percentage of the total FDNB reactive lysine lost) were also similar, although slightly less early Maillard products (51 % vs 63 %) and slightly more advanced Maillard products (49 % vs 38 %) were present in the radioactively labelled heated casein-glucose mixture compared to the heated casein-glucose mixture respectively. Furthermore, the results obtained with factors determined in the present study were similar to those based on the equations of Finot *et al.* (1981), except that the latter estimates of regenerated lysine from the furosine integrator area were 20 - 30 % lower. However, the estimates of regenerated lysine, from the difference between the FDNB reactive lysine and acid hydrolysed lysine values, were approximately 1.5 times higher than those determined from the hydrolysis of ε-DF-L using either the factors of Finot *et al.* (1981) or those from the present work.

The isoelectrically precipitated (IEP) and washed radioactively labelled heated mixture (BR) contained 1.69×10^8 dpm/100 g of dry sample. The total amount of radioactivity added (1 mCi, 4.35×10^{-3} mmol) equated to 2.20×10^8 dpm/100 g (using 2.22×10^9 dpm/mCi). Since 72 % of the glucose had reacted it is assumed that 1.58×10^8 dpm/100 g (2.20×10^8 dpm/100 g x 0.72) would have bound. However, as this was isoelectrically washed the radioactivity should equate to 1.58×10^8 dpm/96.6 g (1.64×10^8 dpm/100 g) since it was assumed that all of the unreacted glucose was washed away, 3.4 g (28 % of the 12 g of glucose per 100 g of BR). As the above estimates are similar, the isoelectric washing procedure was successful in washing away practically all of the unbound glucose and,therefore, unbound radioactivity.

The radioactive glucose added was diluted with 120 g (667 mmol) of cold glucose/1,000 g BR, to give a dilution of 6.52×10^{-6} ($4.35 \times 10^{-3}/667$). Since there was 13.1 mmol ε-DF-L/100 g of IEP BR (12.6 mmol ε-DF-L/100 g of BR before IEP), there was 8.54×10^{-5} mmol U-^{14}C-glucose/100 g IEP BR (13.1 x 6.52×10^{-6}) in the form of ε-DF-L. The percentage of glucose lost as ε-DF-L can be calculated two ways:

(i) From the proportions of bound radioactivity as ε-DF-L from 230 mCi/mmol U^{14}C-glucose: 8.54×10^{-5} x 230 mCi = 1.96×10^{-2} mCi/100 g. Using 2.22×10^{9} dpm/mCi this is equivalent to 4.36×10^{7} dpm/100 g IEP BR from ε-DF-L, i.e. 25.8 % of the total radioactivity bound.

(ii) By calculating the percentage of the total glucose lost which is present as ε-DF-L: Of the total glucose (66.7 mmol/100 g BR), 72 % was lost (48.0 mmol/100 g BR = 50.8 mmol/100 g of IEP BR). As mentioned previously 13.1 mmol/100 g IEP BR was ε-DF-L. This corresponds to 25.8 % of the reacted glucose which is in agreement with the above.

The residual radioactivity not associated with ε-DF-L equates to approximately 74 % of the total radioactivity (1.25×10^{8} dpm/100 g IEP BR). The difference between the total glucose lost and that associated with ε-DF-L was 37.7 mmol/100 g IEP BR (74 %). Arginine and histidine losses only account for a small proportion of the latter, 3.0 mmol/100 g IEP BR (5 %). Therefore, 34.7 mmol/100 g IEP BR (69 %) of the reacted glucose is associated with the portion of FDNB reactive lysine lost not present as ε-DF-L (10.5 mol/100 g IEP BR) and assumed to be in advanced Maillard products.

From the above, specific radioactivities were calculated for (1) early Maillard products - in the form of ε-DF-L; and (2) advanced Maillard products - assuming that all the residual radioactivity is associated with the remainder of the reacted lysine:

(1) Specific radioactivity of ε-DF-L:

$$\frac{4.36 \times 10^{7} \text{ dpm/100 g IEP BR}}{13.1 \text{ mmol/100 g IEP BR}} = 3.33 \times 10^{6} \text{ (dpm/mmol ε-DF-L)}$$

(2) Specific radioactivity of advanced Maillard products (Residual radioactivity):

$$\frac{1.25 \times 10^{8} \text{ dpm/100 g IEP BR}}{11.3 \text{ mmol/100 g IEP BR}} = 11.0 \times 10^{6} \text{ (dpm/mmol FDNB reactive lysine lost not associated in ε-DF-L)}$$

Figure 3.6 Lysine content was measured by the direct FDNB reactive lysine method (2.2.1) and glucose was measured by the dinitrosalicylic acid method (2.2.2). The stoichiometric relationship between the percentage lysine and glucose losses is equivalent to a molar reaction.

Figure 3.6 *The relationship between FDNB reactive lysine and glucose losses (as a % of the amounts in the non-heated mixture) when a casein-glucose mixture was heated to produce varying intensities of browning in an autoclave, and comparison with the stoichiometric relationship .*

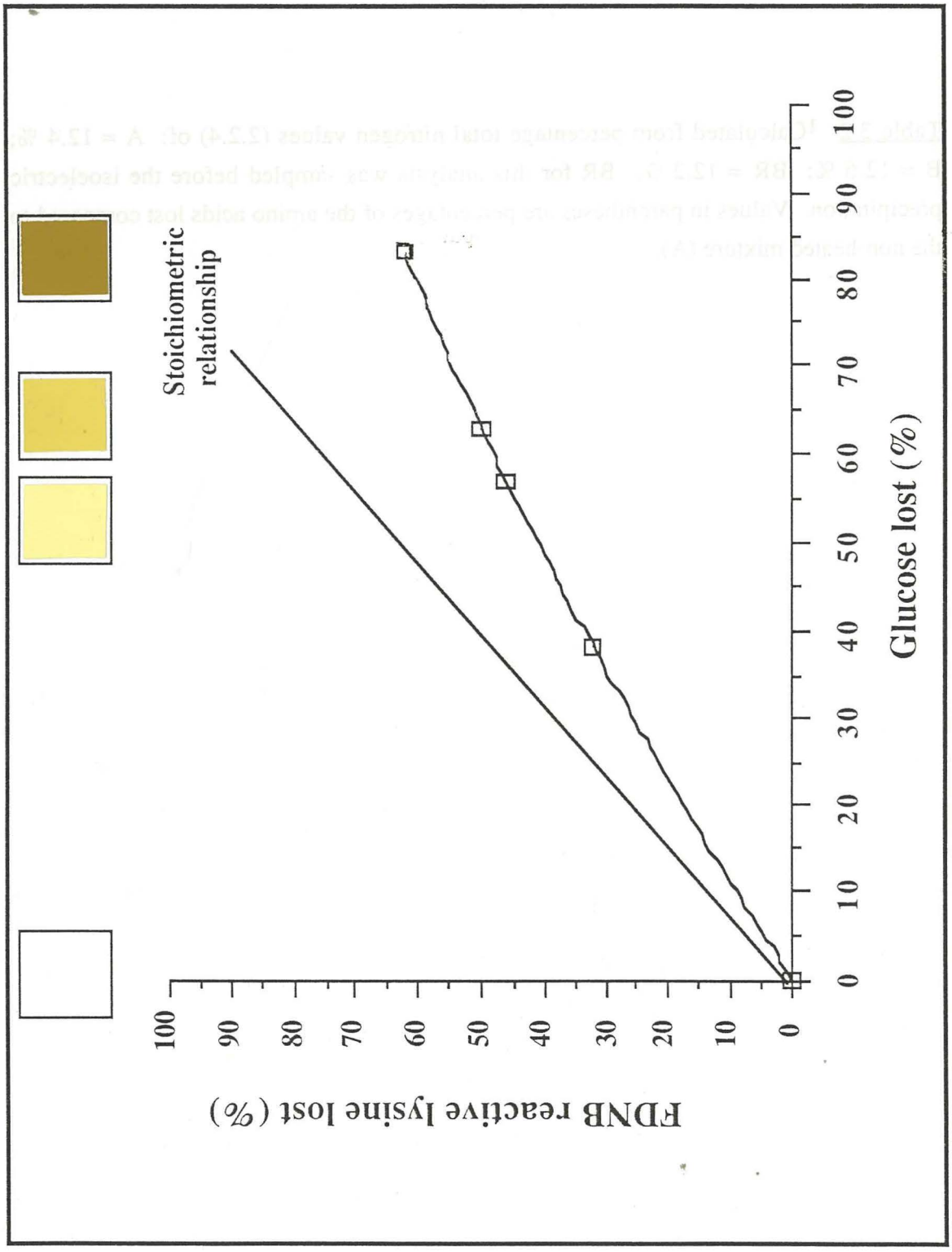


Table 3.2 [1]Calculated from percentage total nitrogen values (2.2.4) of: A = 12.4 %; B = 12.6 %; BR = 12.2 %. BR for this analysis was sampled before the isoelectric precipitation. Values in parentheses are percentages of the amino acids lost compared to the non-heated mixture (A).

Table 3.2 *The amino acid composition of the non-heated (A), heated (B) and radioactively labelled heated (BR), casein-glucose mixtures.*

	Sample (g amino acid/16 g N)[1]		
Amino acid	(A)	(B)	(BR)
Aspartic acid	7.07	7.28	7.06
Threonine	4.19	4.18	4.01
Serine	5.33	5.24	5.34
Glutamic acid	22.58	22.72	22.00
Proline	17.38	17.07	17.65
Glycine	1.85	1.92	1.93
Alanine	3.04	3.06	2.92
Valine	6.59	6.79	6.88
Methionine	2.93	2.90	2.83
Isoleucine	5.22	5.25	5.20
Leucine	9.43	9.79	9.23
Tyrosine	5.64	5.64	6.07
Phenylalanine	5.19	5.38	5.69
Histidine	2.98 (0)	2.89 (3)	2.88 (3)
Lysine	8.07 (0)	5.86 (27)	5.24 (36)
Arginine	4.14 (0)	3.74 (10)	3.58 (14)

Table 3.3 Chemical determinations are reported in Chapter 2. [1]Lysine measured by acid hydrolysis (2.2.3). [2]1-Fluoro-2,4-dinitrobenzene reactive lysine (2.2.1). [3]Dinitrosalicylic acid method (2.2.2). [4]Direct hexokinase method (2.2.2). [5]Furosine expressed in lysine units by using the lysine response factor (162,166 integrator area/nmol of lysine). [6]Estimates using the equations from Finot *et al.* (1981), refer methods (3.2.3). [7]Present estimates from those calculated previously, refer results (3.3.1). BR for these analyses was sampled before isoelectric precipitation. Values in parentheses are percentage losses relative to the non-heated mixture (A).

Table 3.3 *The content of lysine, glucose, and early and advanced Maillard products (expressed as a percentage of the FDNB reactive lysine lost) in the non-heated (A), heated (B) and radioactively labelled heated (BR), casein-glucose mixtures.*

	Casein-glucose mixtures		
Measurements	(A)	(B)	(BR)
Lysine determinations (mmol/100 g):			
Acid hydrolysed lysine (AHL)[1]	42.8 (0)	31.6 (26)	27.4 (36)
FDNB reactive lysine[2]	43.6 (0)	22.7 (48)	19.3 (56)
FDNB reactive lysine lost	0	20.9	24.3
Glucose determinations (mmol/100 g):			
DNS[3]	57.2 (0)	17.8 (69)	17.2 (70)
HEX[4]	53.9 (0)	16.1 (70)	14.4 (73)
Glucose lost (average)	0	38.6	40.0
Determination of the proportions of early and advanced Maillard products:			
Furosine (integrator area/100 g)	0	6.69×10^{11}	6.33×10^{11}
Furosine (mmoles/100 g)[5]	0	4.13	3.90
ε-DF-L (mmoles/100 g)			
3.10 x Fur[6]	0	12.8	12.1
50,262 area furosine/nmol[7]	0	13.3	12.6
Regenerated lysine (mmoles/100 g)			
1.25 x Fur[6]	0	5.16	4.88
1.60 x Fur[7]	0	6.61	6.24
AHL - FDNB	0	8.70	8.10
Lysine lost as early Maillard products, ε-DF-L (%)			
3.10 x Fur/Lysine lost[6]	0	61	50
(50,262 area/nmol)/Lysine lost[7]	0	63	52
Lysine lost as advanced Maillard products, by difference (%)			
Literature[6]	0	39	50
Present[7]	0	37	48
Pigmentation			

3.4 DISCUSSION

Finot and Mauron in 1969 first synthesized the Amadori compound of ε-amino-lysine by reacting α-N-formyl-lysine with glucose and subsequently removing the formyl group. Most other syntheses have followed this procedure. The protection group used in the present study was of a different nature and the synthesis and purification procedures much simplified. Ion-exchange chromatography showed that only traces of other ninhydrin positive compounds were present in the ε-DF-L preparation. However, because only a small amount of ε-DF-L was prepared, the purity of this could not be examined by other methods such as elemental analysis. If the synthesized ε-DF-L was impure the impurities would lead to overestimates of ε-DF-L and regenerated lysine.

Acid hydrolysis of ε-DF-L with and without phenol resulted in different proportions of pyridosine, lysine and furosine areas, and these also differed from Moller (1977 II), and other workers (Finot & Mauron, 1972; and Bujard & Finot, 1978). In these later studies the hydrolysis of free ε-DF-L and that present in protein samples was undertaken using 6 M HCl at 110 °C for 24 hours under reflux. In the present work, hydrolysis was carried out at the same temperature and for the same time but in an evacuated sealed glass tube, instead of by reflux. This was to reduce the oxidation of susceptible amino acids such as threonine, serine and tyrosine (Moore & Stein, 1963; Davies & Thomas, 1973). The acid hydrolysis behaviour of ε-DF-L may, therefore, have been influenced by the method of hydrolysis possibly because of the absence of oxygen in the latter case. This may account for the disagreement between the present values and those from Moller (1977 II). The addition of 0.1 % phenol to the 6 M HCl also affected results. Inclusion of phenol is a recent development (Mason *et al.*, 1980) where the phenol acts as a general antioxidant to aid in the stability of the susceptible amino acids. In the present study this was used in conjunction with the sealed tube method for all samples hydrolysed.

Variation in the proportions of products formed from acid hydrolysis of ε-DF-L was also reported by Finot and Mauron (1972). In their studies the hydrolysis of free ε-DF-L using 6 M HCl resulted in the formation of 50 % lysine and 20 % furosine of the unhydrolysed ε-DF-L, whereas 7.8 M HCl resulted in 44 % lysine and 30 % furosine. It, therefore, appears necessary to evaluate the behaviour of ε-DF-L under the conditions of hydrolysis used. Since in the present study acid hydrolysis was carried out in a sealed

evacuated tube in the presence of phenol, it is considered that the values given are a correct representation of the pyridosine, lysine and furosine areas. Use of these values to calculate the amount of ε-DF-L gave similar results to those obtained using the equations of Finot *et al.* (1981) However, some error may have been introduced by the assumption that the reported differences (Finot *et al.*, 1981) in the acid hydrolysis behaviour of ε-DF-L when it is protein bound or free, apply in the present system. This may explain the higher amounts of regenerated lysine estimated when the present determined values were used rather than those of Finot *et al.* (1981).

It was further noted that measurements of lysine regeneration in protein samples, from the difference between acid hydrolysis values and the FDNB reactive lysine values, were higher than estimates from the furosine recovered areas, using either the equations of Finot *et al.* (1981) or those determined in the present study. These differences may be explained by the fact that early Maillard products prior to the Amadori compound (ε-DF-L), such as Schiff's bases and aldosylamines, are totally converted to lysine on acid hydrolysis (Mauron, 1981), but do not react with FDNB. .Therefore, if these are present they will increase the acid hydrolysis lysine value and when FDNB reactive lysine is subtracted, the amount of regenerated lysine will be overestimated.

The casein-glucose heated mixture (B) and radioactively labelled heated mixture (BR) were similar in their respective extents of the Maillard reaction, resulting in approximately 50 % lysine and 70 % glucose loss. Of the lysine lost approximately 55 % was present as ε-DF-L and 45 % as advanced Maillard products. Of the bound glucose 26 % was associated with ε-DF-L, with the remaining 74 % associated with either the remainder of the bound lysine as advanced Maillard products (a 3:1 ratio) and/or associated with the protein through other mechanisms. Lea and Hannan (1950 II) also reported a higher association of glucose than those offered by the free amino groups which had reacted, and suggested that the observed effect could be either due to a reaction with carbohydrate molecules already attached to the protein, or to glucose decomposition products (caramel) being adsorbed by the protein. In view of this, the calculated residual radioactivity associated with the reacted lysine (11×10^6 dpm/mol) may be an overestimate of the advanced Maillard products present in the heated mixtures.

The only other amino acids to suffer losses due to heat treatment were arginine and histidine. These were much less than lysine losses, being approximately 12 % and 3 %

respectively. The amino acid losses in the present study are similar to those found with the heated casein-glucose systems of Lea and Hannan (1950 III).

Cystine, although not determined in the present investigation, appears to be sensitive to the presence of reducing sugars and further, is heat labile (Miller *et al.*, 1965), Lea and Hannan (1950 III) using casein found that it did not react to any significant degree and, therefore, it is presumed to be of no consequence in the present research. Tryptophan, also not determined, has been suggested to react partly due to the reaction of its amino groups with reducing sugars and also because of the splitting of its indol ring by heat and oxidation (Hurrell & Carpenter, 1981). However, Neilsen *et al.* (1985) has reported it to be relatively stable during food processing and storage and, consequently, suggested that any losses were of minor nutritional significance. The availability of methionine may also be affected by heat treatment as it has a reduced digestibility by the microbial assay (Rao & McLaughlan, 1967). Its involvement may be due indirectly to its oxidation (Hurrell & Carpenter, 1981). However total methionine by performic oxidation, followed by acid hydrolysis, has been reported to be little changed (Lea & Hannan, 1950 III; Sgarbieri *et al.*, 1973). The results obtained in the present study are not of total methionine values as performic acid oxidation was omitted from the procedure. No loss was noted in the heated casein-glucose mixtures.

In summary the heated casein-glucose mixtures can be considered as being early Maillard browned, by the proportions of their early and advanced Maillard products and the intensity of browning.

Chapter 4

THE DIGESTION OF AN EARLY MAILLARD BROWNED CASEIN AND THE ABSORPTION AND EXCRETION OF ITS DIGESTION PRODUCTS IN THE GROWING PIG

The previous Chapter described the production and analysis of an early Maillard browned casein. In the following section, the effects of the formed Maillard compounds on *in-vivo* protein digestion and amino acid absorption are described and discussed. Additionally, work is described which aims to evaluate the faecal and urinary excretion of the absorbed products.

4.1 Introduction

As previously discussed (Chapter 1) the reduction in the nutritive value of proteins in the presence of reducing sugars can be mainly attributed to the destruction of indispensable amino acids by the formation of Maillard products, an overall decrease in protein digestibility, and the formation of absorbed but unavailable compounds which are finally excreted in the urine

Under mild conditions of heat processing, the reduction in nutritive value, measured by a rat growth assay, can usually be fully accounted for by lysine being transformed into early Maillard compounds (Henry *et al.*, 1948; and Henry & Kon, 1950). However, for more severely browned proteins, in addition to losses of amino acids (detected by acid hydrolysis), faecal nitrogen digestibilities also decreased, although not sufficiently to explain the decreased nutritive values found by the rat growth assays (Evans & Butts, 1949; and Horn *et al.*, 1952).

This discrepancy is partly explained by the influence of the microbial population in the large intestine on estimates of nitrogen digestibility. This was demonstrated by Nesheim and Carpenter (1967), who found differences in the digestibility of heat-damaged cod muscle fed to caecectomized or normal chicks with exteriorized ureters - for the separation of urine and faeces. Lower digestibility values were found for the caecectomized birds. These authors concluded that degradation of nitrogenous compounds occurred in the caecum. They suggested that the micro-organisms in the large intestine may digest material entering from the terminal ileum, leading to overestimates of faecal nitrogen digestibility. This was supported by Ford *et al.* (1967) who also found that the availability of amino acids and nitrogen were overestimated when using faecal digestibility measurements. A further study by Salter and Coates (1971) on the influence of the alimentary tract microflora on protein digestion, using conventional and germ free chicks, also demonstrated that faecal digestibilities overestimated amino acid and nitrogen absorption. This phenomenon is now known to be important in several species of simple-stomached animals, including pigs (Moughan & Smith, 1982).

In a further development Varnish and Carpenter (1975) compared ileal and faecal digestibility coefficients of heated pure proteins and non-heated propionylated proteins. Ileal digestibility coefficients were found to give a far better estimate of amino acid availability, determined by a chick growth assay, than did faecal digestibilities. However, the decrease in digestibility based on ileal measurements still did not fully account for the reduction in nutritive value.

In spite of the superiority of the ileal method over the faecal one, few attempts have been made to study the digestion of Maillard products or heat-treated proteins, using this approach. In a recent study (Plakas *et al.*, 1985) a lower apparent ileal digestibility of lysine and other amino acids was demonstrated when heat-damaged fish protein was fed to rainbow trout. The decrease in the apparent ileal digestibility may be attributed to three causes: the formation of enzymatically resistant cross linkages (Clark & Tannenbaum, 1970); competition of Maillard products with amino acids and small peptides for their absorption sites (Lee *et al.*, 1977 A) and enhanced losses of endogenously secreted proteins (Schneeman & Dunaif, 1984).

Evidence for the formation of absorbed but unavailable compounds was obtained by Valle-Riestra and Barnes (1970). They fed radioactively labelled heat-damaged albumin

to rats and demonstrated that radioactivity was excreted in the urine above control levels. Further evidence for the urinary excretion of absorbed but unavailable compounds resulting from Maillard heat-damaged proteins, has also been reported by Mori and Nakatsuji (1977), Mori *et al.* (1980) and Finot and Magnenat (1981). Absorbed but unavailable compounds have also been reported on feeding severely heat-damaged pure proteins (Ford & Shorrock, 1971) to rats, with excretion of small peptides in the urine.

The overall objective of the present study in the following Chapter, therefore, was to investigate the digestion of protein by growing pigs fed an early Maillard browned casein. In addition, the absorption and subsequent excretion of the digestion products were examined. The specific aims were:

(1) - To determine the ileal and faecal digestibility of amino acids and Maillard compounds, and to compare the absorption of amino acids from non-heated and heated casein-glucose mixtures, and to describe the uptake of Maillard compounds from the gut.

- To assess the influence of the large intestine microbial population on the digestion and absorption of amino acids and Maillard compounds from the gut.

- A secondary aim was to determine the dry matter and chromium recoveries and the retention times of chromium along the gastro-intestinal tract. Furthermore, digestion in sections of the small intestine was examined.

(2) - To determine whether the formation of limit peptides containing Maillard products in the ileal digesta, could account for decreased digestion of protein.

(3) - To determine the fate of radioactively labelled Maillard compounds (i.e. uptake from the small intestine, disappearance from the large intestine, excretion in faeces and excretion in urine).

(4) - To estimate the urinary excretion of the absorbed Maillard products and evaluate whether these may be associated with amino acids in small peptides which are absorbed but cannot be utilized which are, therefore, finally excreted in the urine.

4.2 Experimental

4.2.1 Diet formulation

Three semi-synthetic diets containing either a non-heated or heated casein-glucose mixture (described in Chapter 3) as the sole protein sources, were formulated to satisfy the nutrient requirements of the young growing pig (Agricultural Research Council (ARC), 1981). Each diet consisted of two parts (Table 4.1) stored separately and mixed prior to feeding. The basal mixture, common to all diets, was mixed in four 25 kg batches, using a large Hobart-mixer. All ingredients were previously passed through a 1 mm mesh sieve. The maize oil was added last to ensure an even distribution of chromic oxide in the feed. The chromic oxide was added as an indigestible marker to allow the calculation of digestibility coefficients.

The three experimental diets were:

DIET A (Non-heated diet): This contained 30 % of the non-heated casein-glucose mixture (A) and 70 % of the basal mixture, to provide a diet with a final crude protein concentration of 264 g casein/kg.

DIET B (Heated diet): This contained 32.5 % of a lysine and glucose supplemented casein-glucose heated mixture and 68.5 % of the basal mixture. The casein-glucose mixture (B) was supplemented with the lysine and glucose lost during heat treatment. As 50 % of the total lysine and 70 % of the total glucose had reacted, the equivalent amount of free lysine and glucose were added to this mixture. The total amount of lysine and glucose added equaled 3.7 g per 30 g of heated mixture. Consequently, for every 100 g of diet B, calculated to be required by the pigs in this treatment, 103.7 g was fed, i.e. 33.7 g of supplemented casein-glucose mixture B, and 70 g of the basal mixture to provide a diet with a final crude protein concentration of 264 g casein/1.037 kg.

DIET BR (Radioactively labelled heated diet): This contained 30 % of the radioactively labelled heated casein-glucose mixture (BR) and 70 % of the basal mixture to provide a diet with a final crude protein concentration of 264 g casein/kg.

The major minerals provided by the diet met the requirements for the young growing pig (ARC, 1981) (Table 4.2). The amino acid balances (relative to lysine) of diets A and B were similar (Table 4.3) and comparison with the recommended balances (ARC, 1981

Table 4.1 [1]Tasmix, pig creep vitamin mineral premix (Tasman Vaccines Ltd, Auckland, NZ.). [2]The supplements were premixed into the heated mixture (B) and the weight of mixture added to the basal mixture adjusted accordingly for the extra weight of supplements. Glucose was supplemented for a 70 % loss from the 120 g/kg of mixture, and lysine was supplemented for a 50 % loss of the 63 g/kg from the mixture (these were estimated previously in Chapter 3). [3]The nutrient content was expressed to an equivalent fed, i.e. accounting for the extra weight of supplements in diet B. The amino acid levels and their determination are described in Chapter 3 (Table 3.3). [4]These lysine values do not include the regenerated lysine from ε-DF-L. [5]Arginine, aspartic acid, serine, glutamic acid, proline, glycine and alanine.

and Moughan & Smith, 1984) indicates that the diets were likely to be first limiting for threonine. However, it should be noted that overall the protein was well balanced.

Table 4.1 *Ingredient compositions of the basal mixture and casein-glucose mixtures used to prepare the experimental diets, and the amino acid compositions of the casein-glucose mixtures.*

INGREDIENT	Composition (g/kg air-dry weight)		
	Diet A	Diet B	Diet BR
(i) *Basal mixture*			
Maize starch	521.6	503.0	521.6
Sucrose	50.1	48.3	50.1
Dicalcium phosphate	40.0	38.6	40.0
Maize oil	40.0	38.6	40.0
Purified cellulose	35.1	33.8	35.1
Vitamins, trace minerals[1]	5.0	4.8	5.0
Potassium chloride	4.0	3.9	4.0
Chromic oxide	3.0	2.9	3.0
Magnesium sulphate (anhydrous)	1.2	1.2	1.2
(ii) *Casein-glucose mixtures*			
Non-heated mixture (A)	300	-	-
Heated mixture (B)	-	289.3	-
Radioactively labelled heated mixture (BR)	-	-	300
Supplements[2]			
Glucose (anhydrous)	-	24.3	-
Lysine (mono-hydrochloride)	-	11.4	-
NUTRIENT[3] - Amino acid content	(g/kg)	(g/1.037 kg)	(g/kg)
Lysine	18.8	18.5[4]	9.6[4]
Methionine	6.8	6.6	6.7
Tryptophan	ND	ND	ND
Histidine	6.9	6.9	6.8
Phenylalanine + tyrosine	25.2	25.2	28.0
Threonine	9.7	9.6	9.5
Leucine	21.9	22.6	21.9
Isoleucine	12.1	12.1	12.4
Valine	15.3	15.7	16.4
Non essential amino acids[5]	142.6	136.8	136.6

Table 4.2 [1]Agricultural Research Council requirements (1981). The mineral composition was calculated from the chemicals added to the basal mixture. Sodium values also include that from the sodium-caseinate in the casein-glucose mixtures.

Table 4.3 [1]Moughan and Smith (1984). [2]Agricultural Research Council requirements (1981). [3]Methionine + cystine are usually reported together, but since cystine was not determined in the present study the ARC values have been adjusted for methionine only (50 % of the combined methionine + cystine value). [4]Arginine, aspartic acid, serine, glutamic acid, proline, glycine and alanine. These values were calculated from Table 4.1.

Table 4.2. *The macro mineral composition (g/kg air-dry weight). of the experimental diets and comparison with recommended allowances for the young pig.*

Mineral	Composition	Recommended level (ARC)[1]
Calcium	14.0	8.0
Phosphorus	7.1	7.0
Sodium	3.2	1.0
Chloride	3.1	1.5
Potassium	2.1	2.5
Magnesium	0.3	0.4

Table 4.3. *The determined amino acid balance relative to lysine of the experimental diets and comparison with recommended ideal amino acid balances for the young pig.*

Amino acid	Dietary balance		Ideal balance (M&S)[1]	Ideal balance (ARC)[2]
	Diet A	Diet B		
Lysine	100	100	100	100
Methionine[3]	36	36	-	25
Tryptophan	ND	ND	59	60
Histidine	37	38	34	33
Phenylalanine + tyrosine	134	136	101	96
Threonine	52	52	59	60
Leucine	116	122	89	100
Isoleucine	64	65	46	54
Valine	81	85	66	70
Non essential amino acids[4]	759	739	693	857

4.2.2 Animals and housing

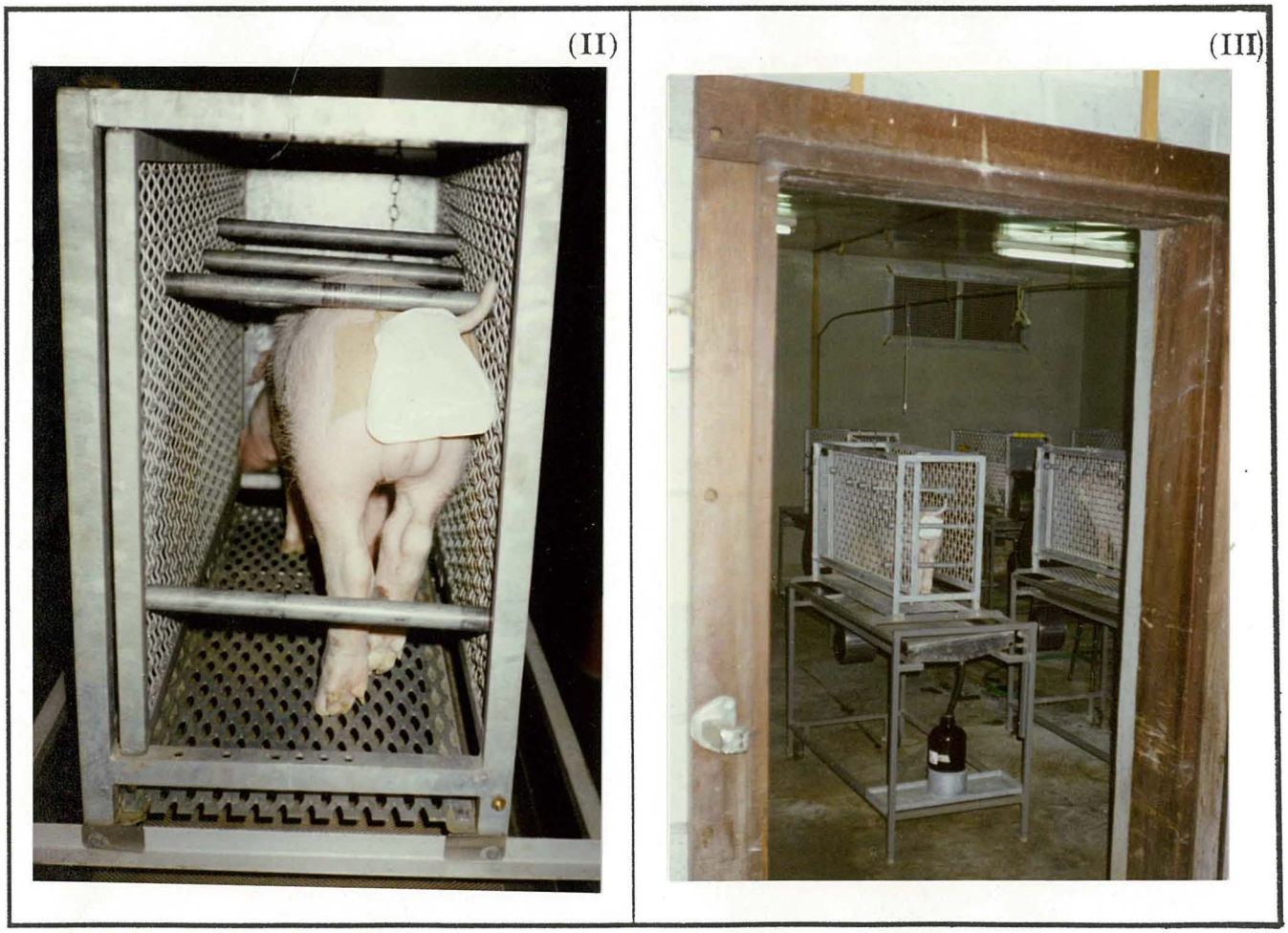
Eight Landrace X Large White entire male pigs were selected from the Massey University Pig Research Unit to satisfy two criteria, firstly a body weight of approximately 10 - 12 kg and secondly a common litter origin for pairs of pigs to minimize genetic variability (four pigs were selected from one litter and four from another). Pigs were individually housed in metabolism cages in an environmentally controlled room (ambient temperature 23 ± 1 °C).

The metabolism cages (Figure 4.1) were designed for the total and separate collection of food spillages, urine and faeces. The pigs were constrained from turning around in the cage while the trial was in progress.

To ensure complete and uncontaminated collection of faeces and urine, faecal material was collected in bags (Figure 4.1 II) placed over the pig's anus. All hair was removed around the pig's rear by shaving, and the exposed area dried with alcohol. A 32 mm stomahesive wafer with flange (Convatec, Squibb) was molded to the pig's rear around the anus and glued into position using superglue. The collection apparatus was reinforced by placing two strips of elastoplast tape over the wafer. The 32 mm collection bag was firmly clipped into position.

Figure 4.1 (I) Side profile of the metabolism cage with all apparatus in position showing feeding troughs, pig constraints and urinary collection apparatus. (II) End profile showing the position of the faecal collection bag and the wire netting over the urinary collection tray. (III) Room layout during the trial.

Figure 4.1 *The metabolism cages for the complete and separate collection of urine and faeces.*



4.2.3 Procedure

General conduct of trial

There was a 3 day period prior to the beginning of the trial to allow accustoming to the new environment. During this period the pigs were initially fed a pelleted weaner ration and then gradually introduced to the experimental diets. Eight pigs were fed the experimental diets for 26 days. Four pigs received the control non-heated diet (A) and four pigs the heated diet (B) at a daily intake level of 8 % metabolic body weight (MBW, $kg^{0.75}$). The food was given twice daily (8:30 am and 4:30 pm). Each of the test diets was mixed thoroughly with water (approximately 3.0 ml/g of diet) to a consistent creamy mixture. After each meal the pig's feet and snout were cleaned using a damp muslin cloth, the cages were wiped and the room washed out with water containing disinfectant. All food refusals were collected into preweighed tin foil trays, oven-dried, cooled and then weighed.

At the beginning of the trial the pigs were weighed prior to their morning meal and the dietary intakes adjusted to 8 % MBW. The pigs were reweighed and the intakes adjusted on day 7 and 13, to ensure maximum consumption of the test diets. The level of intake at day 13 was maintained until the end of the trial, day 26. On the morning of day 21, the pigs were weighed and those pigs normally receiving diet B were fed the radioactively labelled diet BR (at the same intake levels) for the morning meal only, subsequent meals were of the usual diet B. This pulse (BR) - chase (B) feeding regime was to track the radioactivity excretion pattern in the faeces and urine.

Faecal collections, over days 21 - 25 (5 days), were made at 12 hour intervals. At collection each bag was removed, weighed and the bag and contents frozen (-20 °C) awaiting further processing.

Urinary collections, over days 21 - 24 (4 days), were made every 6 hours for the first 36 hours, followed by one 12 hour and two 24 hour collections. Before each collection the netting and tray were sprayed with a small amount of distiled water and the washings collected into preweighed winchester bottle. The winchester bottles contained 4 M HCl (2.5 % w/w of urine, assessed from the previous collection), ensuring preservation and inhibition of bacterial growth. Each urinary sample was weighed, filtered through a fine meshed nylon cloth and stored chilled in plastic bottles.

On the morning of day 26, exactly twelve hours after the previous night's meal, each pig was given its total daily ration, in equal portions, over 6 hours (7 meals). Those pigs which had previously received diet B were fed diet BR at these hourly feeds. One hour after the seventh feed (on the seventh hour) the pig was removed from its cage, anaesthetized (using halothane carried in oxygen) and the liveweight recorded.

The jugular and portal veins were exposed and samples of blood were collected in 50 ml centrifuge tubes and allowed to clot at room temperature for 45 minutes. The clot was separated by centrifuging at 4,500 rpm for 10 minutes. The serum was split into five 2 ml fractions and a remaining fraction. The samples were immediately frozen. Subsequent to the blood sampling, each animal was killed by an intracardial injection of sodium-pentobarbitone (recommended dose 1 ml/kg body weight). This method of euthanasia is considered (Low, 1981) to minimize the loss of cells into the gut at slaughter.

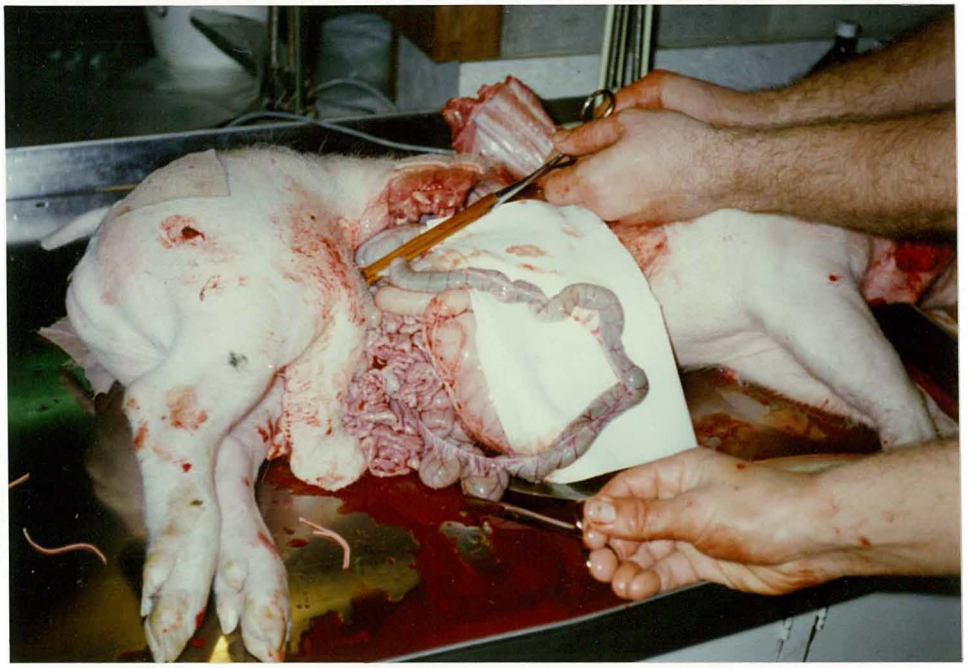
Immediately after slaughter, the abdomen was opened and ties were placed just before the cardiac sphincter, immediately after the pyloric sphincter, at the end of the pancreatic duct, at the caecal/colonic junction and at the end of the rectum. The ileocaecal junction was located, the terminal ileum clamped, and a second clamp was placed 20 cm proximal to this (Figure 4.2 I). This terminal piece of intestine (20 cm) was then removed, the ends carefully washed, blotted dry and the contents gently flushed out with isotonic saline into a plastic beaker (Figure 4.2 II). These ileal digesta samples were immediately frozen. The procedures were carried out swiftly as it was necessary to remove the gastro-intestinal contents before cells sloughed from the mucosa into the lumen. Extreme care was taken not to handle the intestine directly. It was held by the mesentery at the top end and gently supported at the lower end.

The remaining viscera were then removed and placed on a tray of ice. The stomach contents were weighed, immediately bagged and frozen. The stomach was cut open and residual material washed out with isotonic saline. The stomach tissue was blotted dry, weighed and frozen. Mesentery was dissected from the small intestine and the intestine carefully laid-out on a bench. The intestine was divided into six portions of approximately equal length. The contents of each section were flushed out with isotonic saline into plastic bags and frozen. The respective portions of intestinal tissue were bagged and also frozen.

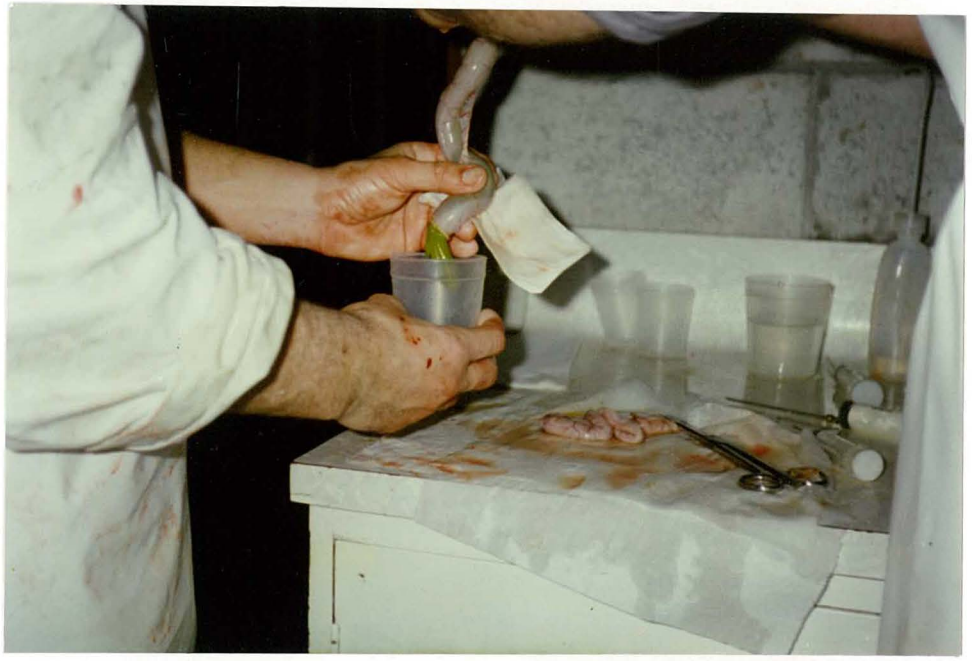
Figure 4.2 (I) Removal of the final 20 cm of ileum. The top clamp is situated at the ileo-caecal junction and the scissors leading toward the bulk of the small intestine. (II) The ileal digesta were flushed from the terminal ileum with isotonic saline into a plastic beaker. The green colour is due to the presence of the indigestible chromic oxide marker.

Figure 4.2 *Sampling of digesta from the terminal ileum of the pig by the slaughter method.*

(I)



(II)



4.2.4 Sample processing

All homogenizing was carried out using an Ultra-Turrax homogenizer at full speed for the times specified. The homogenates were centrifuged at 27,000 g for 10 minutes (0 - 10 ^{o}C) and the supernatants filtered through glass wool. All operations were carried out on ice.

Gastro-intestinal contents

The stomach contents were thawed, weighed and homogenized (1 x 1 min pass). A portion of the homogenate from each pig was immediately frozen and from the remainder, an amount corresponding to an equal proportion of the total sample for each pig was pooled across pigs according to group (A or B), freeze-dried, weighed, ground to a fine powder and frozen.

The small intestinal contents were freeze-dried, ground and weighed. Half of the material in each sample was pooled across pigs according to intestinal section (1 - 6) and group (A or B) and frozen.

The terminal ileal digesta samples were thawed, weighed and homogenized in an equal volume of isotonic saline (1 x 30 s pass). A portion of the homogenate was freeze-dried, weighed, ground and frozen. An equal portion of the freeze-dried ileal digesta sample from each pig was pooled across pigs according to group (A or B) and also frozen.

Tissues

The stomach tissue was thawed, blotted dry and weighed. The mucosa was peeled off, chopped up and passed through a hand mincer. The mince was homogenized in five times its weight of isotonic saline (2 x 1 min passes), centrifuged, filtered and a portion of the supernatant stored frozen. From the remaining supernatant equal proportions of the total sample from each pig were pooled across pigs according to group (A or B), freeze-dried, weighed, ground and frozen.

Tissues from each section of the small intestine were also thawed, blotted dry and weighed. The mucosa for each section was removed by scraping with a glass slide. The mucosa samples were homogenized in an equal weight of isotonic saline containing 2 % Triton X-100 (1 x 30 s pass). The homogenates were solubilized on ice for 1 hour, with occasional stirring, centrifuged and filtered. From the supernatants equal proportions of

the total sample from each pig were pooled across pigs according to intestinal section (1 - 6) and group (A or B), freeze-dried, weighed, ground and frozen.

Urine

The separate urine samples for each pig in group A were pooled per individual, mixed and the samples frozen. Half of each sample for each pig in group B was frozen, the remaining halves were pooled for each pig. An equal portion of the urine samples from the total collection from each pig were pooled across pigs according to group (A or B) and also frozen.

Faeces

All faeces samples were freeze-dried, weighed and ground. The separate faeces samples for each pig in group A were pooled per individual, mixed and frozen. Half of each sample for each pig in group B was frozen, the remaining halves were pooled for each pig, mixed and also frozen.

4.2.5 Laboratory analysis

A summary of the analyses conducted on samples, is given in Table 4.4. The standard analytical techniques are described in detail in Chapter 2.

Gel chromatography of urine and ileal digesta

Sample processing

Urine samples Exactly 20 ml of the pooled urine (across pigs) from each group were transferred to preweighed round bottomed flasks, freeze-dried and weighed. The residue was suspended in 0.2 M acetic acid, quantitatively transferred to a 5 ml volumetric flask and made-up to volume.

Freeze-dried ileal digesta samples Exactly 300 mg of the freeze-dried pooled (across pigs) digesta from each group were weighed into 5 ml beakers to which 4 ml of 0.2 M acetic acid was added and the suspension adjusted to pH 2.6 using glacial acetic acid. This was stirred occasionally, left to stand overnight at room temperature (to ensure maximum solubilization) and quantitatively transferred to a 5 ml volumetric flask and made-up to volume.

Sephadex G-15 chromatography

A previously used Sephadex G-15 superfine gel was firstly regenerated with four successive washings of each of the following solutions in a large buchner funnel (0.5 M NaOH, 0.5 M NaCl, 0.5 M HCl). Residual solution was removed by placing under vacuum. After each washing residual solution was removed by rinsing with deionised water. The column was then equilabrated with four washings of 0.2 M acetic acid (elution buffer) and resuspended to a slurry. The gel was degassed in a buchner flask under vacuum and then carefully poured into a column (620 cm in length; internal radius 1.25 cm) with the aid of a glass rod to minimize turbulence. The gel was settled overnight at a constant flow rate and the loader placed on top of the settled gel for the application of samples. The column was calibrated using a mixture of molecular weight (MW) markers (bovine serum albumin > 30,000, glutathione 612, leucine 131 and urea 60 MW).

The 5 ml of suspended material from the urine and ileal digesta were centrifuged at 3,000 rpm for 10 min. A 3 ml aliquot of the supernatant was removed and chromatographed on the Sephadex G-15 superfine column, run in 0.2 M acetic acid. Approximately 10 ml (200 drops) was collected in each tube for the first ten fractions, then approximately 5 ml (100 drops) for the remaining eluate. These were collected at a flow rate of 20 ml/hr. Fractions were analyzed for: absorbances at 280 and 420 nm on a HP 8452A spectrophotometer; the distribution of ninhydrin positive compounds (2.2.9); and ^{14}C radioactivity (2.2.5) to identify the distribution of Maillard products from diet BR. Poolings were then made at appropriate intervals, according to the above elution patterns, and aliquots removed for the analysis of ^{14}C radioactivity (2.2.5), and ε-DF-L and amino acid content by acid hydrolysis (2.2.3).

Table 4.4 [1]The following analytical techniques are described in Chapter 2. ^{14}C = radioactivity determination (2.2.5), **aa** = amino acid analysis (2.2.3), **Cr** = chromium (2.2.6), **N** = total nitrogen (2.2.4), **Creat** = creatinine (2.2.8) and **FDNB** = 1-fluoro-2,4-dinitrobenzene reactive lysine (2.2.1). [I]Superscripts denote analyses carried out on individual samples. [P]Superscripts denote analyses carried out on pooled samples according to group (A or BR).

Table 4.4 *Summary of the chemical and physical analyses.*

Sample		Techniques[1]
Stomach[P]:	Contents	^{14}C, aa, Cr, N.
	Mucosa	^{14}C, aa.
Small intestine (1 - 6)[P]:	Contents	^{14}C, aa, Cr, , N.
	Mucosa	^{14}C, aa, N.
Ileal digesta[I]:		^{14}C, aa, Cr, N, FDNB. Ileal fractionation[P].
Urine[I]:		^{14}C, aa, N, Creat, Urine fractionation[P].
Faeces[I]:		^{14}C, aa, Cr, N.
Serum[I]:	Portal	^{14}C, aa.
	Jugular	^{14}C, aa.

4.2.6 Calculations of digestibility coefficients, digesta retention times and Maillard product concentrations

Apparent dry matter, amino acid, nitrogen and Maillard product digestibility coefficients in the gastro-intestinal contents and faeces were calculated, by reference to an indigestible marker (chromic oxide, Cr) using the following equation:

EQ (1)

$$\text{Digestion of compound (\%)} = \frac{\text{diet (mg/g Cr)} - \text{sample (mg/g Cr)}}{\text{diet (mg/g Cr)}} \times \frac{100}{1}$$

Apparent faecal digestibility coefficients were also calculated from total collection using the following equation:

EQ (2)

$$\text{Digestion of compound (\%)} = \frac{\text{dietary intake (g/day)} - \text{faecal excretion (g/day)}}{\text{dietary intake (g/day)}} \times \frac{100}{1}$$

The mean retention time of digesta was calculated by reference to the retention time of the indigestible chromic oxide marker using the following equations (Wilson & Leibholz, 1981):

EQ (3)

$$\text{Mean retention time (min)} = \frac{\text{weight of digesta (g)}}{\text{flow rate of digesta (g/hr)}} \times \frac{60}{1}$$

where

$$\text{flow (g/hr)} = \frac{\text{rate of marker administration (g/hr)}}{\text{marker in sample (g/g)}}$$

The concentrations of Maillard products were determined by measurement of the ε-DF-L and radioactivity contents in the samples taken. The following calculations which were used are reported in detail in Chapter 3.

In the stomach contents, ε-DF-L was calculated assuming it was protein bound (50,262 integrator area of furosine/nmol of ε-DF-L). In the small intestine, ε-DF-L was calculated assuming it exists as a 50/50 combination of both protein bound and free (40,838 area furosine/nmol ε-DF-L). In all other samples such as faeces, mucosa, blood and urine, ε-DF-L was calculated assuming it was present as the free molecule (31,414 area furosine/nmol of ε-DF-L).

The regenerated lysine values from ε-DF-L (calculated from furosine) contribute to the lysine area after acid hydrolysis. Regenerated lysine was estimated using the above assumptions for the behaviour of ε-DF-L: protein bound (regenerated lysine = 1.6 x furosine area); combination (regenerated lysine = 2.4 x furosine area); and free (regenerated lysine = 3.2 x furosine area). The regenerated lysine (RL) was subtracted from the acid hydrolysis lysine (AHL) to provide an estimate of the actual lysine (AL) content in samples.

The amounts of advanced Maillard products were determined by calculating the residual radioactivity. The latter was determined by subtracting the radioactivity due to ε-DF-L (3.33×10^9 dpm/mol) from the total radioactivity measured. Residual radioactivity

expressed in lysine molar units was calculated from the specific radioactivity (11.0×10^9 dpm/mol of reacted lysine).

4.2.7 Statistical analysis

The respective data sets were subjected to statistical analysis with routines being performed using a statistical analysis systems package (SAS), run on a mainframe computer (Primos network).

The variances around the respective means were tested initially for homogeneity using a Bartlett's test (Snedecor & Cochran, 1982). When conducting analysis of variance it is necessary that the variances around the means be homogeneous.

Ileal and faecal digestibility data were fitted to a linear model which included terms for diet (A or B), digestibility type (ileal or faecal) and the interaction (diet x type):

$$Y = \mu + \text{diet} + \text{type} + \text{diet x type} + \varepsilon$$

Levels of significance were determined by reduction of sums of squares (Snedecor & Cochran, 1982). When a significant interaction was found the data were then analysed within digestibility type.

Differences in faecal amino acid digestibility (as determined by either total faecal collection or by reference to an indigestible marker, chromic oxide), dry matter contents in sections of the gastro-intestinal tract (between diets) and urinary amino acid excretion (between diets), were examined using a one-way analysis of variance.

The overall standard errors were calculated from the overall standard deviation divided by the square root of the average number of observations per treatment.

4.3 Results

4.3.1 The digestion and absorption of amino acids and Maillard compounds, determined at the terminal ileum or over the entire digestive tract

The animals consumed their respective diets readily and food refusals were minimal. The quantitative uncontaminated collection of faeces using the plastic collection bags was successful. This method proved to be easy and efficient with the bags remaining in position throughout the collection period.

Apparent faecal amino acid and nitrogen digestibility coefficients were determined by reference to the indigestible marker and by total collection. Although statistically significant differences were found between the two methods (Appendix 4.1), the differences were very small practically (mean difference over all amino acids and nitrogen was < 1 % unit). As a result only the digestibility coefficients determined using the marker method, are reported here.

When the apparent ileal and faecal amino acid digestibility data were subjected to analysis of variance, a significant ($P < 0.05$) interaction (diet x source) was found for several amino acids and nitrogen (Appendix 4.2). This means that there were significant differences between the two diets but that the magnitude of the differences differed with the type of measurement (ileal or faecal). The data for each amino acid were then analysed within diet and source and the overall mean digestibility coefficients are presented in Table 4.5.

Significant ($P < 0.05$) differences between the two diets (A & B) for digestibility determined at the end of the ileum were found for threonine, serine, glutamic acid, proline, valine, methionine, isoleucine, histidine, lysine, arginine and nitrogen. The release from protein and uptake of these amino acids was lower for diet BR. Although the effect of diet was statistically significant for all of these amino acids, only the differences for lysine (39.4 % units), threonine (4.1 % units) and arginine (6.8 % units) and also nitrogen (5.6 % units) were of practical significance. The mean ileal amino acid digestibility coefficients for diet BR were always lower than those for diet A, except for

aspartic acid and glycine. With the exclusion of lysine and glycine, the amino acids from diet BR were less digestible (by 2.5 % units) than those from diet A.

When digestibility was determined over the entire digestive tract, the only statistically significant difference found ($P < 0.01$) was for nitrogen, where the digestibility coefficient was higher for diet A. The faecal amino acid digestibility coefficients tended to be slightly lower for diet BR than diet A. It is notable that the large difference found for the ileal digestibility of lysine was not detected using the faecal method.

Statistically significant ($P < 0.05$) differences between ileal and faecal digestibility coefficients (within each diet) were found for nitrogen and for most of the amino acids (except for methionine, leucine and tyrosine for diets A and BR, phenylalanine for diet BR and arginine for diet A). Faecal values were generally higher than their ileal counterparts and there was a tendency for the ileal/faecal differences to be greater for diet BR compared to A. It was also noted that the least digestible amino acids had the greatest ileal/faecal differences (correlation coefficient = 0.99).

Lysine digestibility coefficients were determined using lysine levels from acid hydrolysis (which contained lysine regenerated from the hydrolysis of ε-DF-L). The levels of FDNB reactive lysine and ε-DF-L (expressed as furosine in lysine equivalents) in ileal digesta were also determined and are given in Table 4.6. These values allow not only calculation of the levels of regenerated lysine (from furosine) and, therefore, actual lysine present in ileal digesta, but also the proportions of actual lysine present as either C-terminally or internally bound (FDNB reactive lysine) and also as either N-terminally bound or free (Actual lysine - FDNB reactive lysine), given in Table 4.6. The digestibility of acid hydrolysed lysine and actual lysine, determined at the terminal ileum of pigs given diets A and BR are shown in Table 4.7.

The data in Table 4.6 show that the amount of lysine in terminal ileal digesta from pigs fed diet BR was considerably higher than for diet A, with approximately double the FDNB reactive lysine and 8 times the acid hydrolysed lysine. The amount of actual lysine (taking into account the regenerated lysine from the hydrolysis of ε-DF-L) was 5.4 times higher. The lysine which was either N-terminally bound or free was 13 times higher for diet BR compared to diet A. There was also a much lower proportion of the actual lysine content present which was either C-terminally or internally bound for diet BR (25 %)

compared to diet A (70 %). The amounts of FDNB and actual lysine in the digesta not only showed that there was more lysine present in the ileum of pigs fed diet BR, but that the distribution of the lysine was also altered.

For diet A the acid hydrolysed lysine (which is actual lysine) digestibility coefficients (reported in Table 4.7) were high. Those for diet BR were considerably lower, although the FDNB reactive lysine values were close to those found for diet A. The digestibility of actual lysine for pigs given diet BR was similar to the acid hydrolysed lysine digestibility. However, depending on the assumption made concerning the form of ε-DF-L present in the ileum (either bound or free), the ileal digestibility coefficients for actual lysine may range from 53 to 71 %.

The ileal and faecal digestibility coefficients for ε-DF-L, total radioactivity and residual radioactivity (total radioactivity - radioactivity attributable to ε-DF-L) are given in Table 4.8, as well as values for the amounts of material broken-down in the large intestine, expressed as proportions of the amounts of material entering the organ.

The ileal digestibility of the dietary Maillard compounds was considerably lower than the overall digestion of amino acids and nitrogen in diet BR. The ileal digestibility of ε-DF-L was particularly low (49 %), being much lower than that for the compounds associated with the residual radioactivity component (77 %). Once again depending on the assumption made concerning the form of ε-DF-L (either free or bound), the ileal digestibility coefficient for ε-DF-L may range from 34 to 58 %, with the residual radioactivity estimate ranging from 73 to 82 %. The data were very variable, indicating large inter-animal differences in the degree of uptake of the Maillard compounds. As found previously for lysine, the digestibility of the Maillard compounds determined over the entire digestive tract was high, indicating considerable metabolism of the compounds by the hindgut microflora. The ε-DF-L appeared to be particularly well metabolized in the large intestine in relation to the compounds found in the residual radioactivity fraction. The ε-DF-L accounted for about 45 % of the total radioactivity determined at the terminal ileum but only around 14 % in the faeces.

Table 4.5 [1]Amino acids: **Asp** = aspartic acid, **Thr** = threonine, **Ser** = serine, **Glu** = glutamic acid, **Pro** = proline, **Gly** = glycine, **Ala** = alanine, **Val** = valine, **Met** = methionine, **Ile** = isoleucine, **Leu** = leucine, **Tyr** = tyrosine, **Phe** = phenylalanine, **His** = histidine, **Lys** = lysine, **Arg** = arginine; and **Nit** = nitrogen. [2]Sample source, **IL** = ileum and **F** = faeces . [3]Statistical level of significance between diets and between sources: **NS** not significant, * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$). The number of observations per diet is 4, except: [4]This glutamate value does not include pig # 2 as its concentrations in ileal digesta were more than triple the others in group A; and [5]This acid hydrolysis lysine value does not include pig # 3 as its concentrations in ileal digesta were more than triple the others in group B.

Table 4.5 *The mean apparent ileal and faecal digestibility of amino acids and nitrogen in pigs fed the non-heated diet (A) or the heated diet (BR).*

AA[1]	Source[2]	Digestibility (%) A	Digestibility (%) B	Overall standard error	Level of significance[3] Diet	Level of significance[3] Source
Asp	IL	89.05	89.33	0.69	NS	A **
	F	96.33	94.90	0.79	NS	B ***
Thr	IL	84.95	80.83	0.96	*	A ***
	F	96.95	95.83	0.62	NS	B ***
Ser	IL	89.68	86.88	0.41	**	A ***
	F	97.53	96.43	0.55	NS	B ***
Glu	IL	95.05[4]	93.30	0.21	**	A ***
	F	98.65	98.03	0.27	NS	B ***
Pro	IL	96.55	94.28	0.22	***	A ***
	F	99.28	98.87	0.14	NS	B ***
Gly	IL	49.65	54.30	8.00	NS	A **
	F	92.72	91.53	1.64	NS	B *
Ala	IL	89.45	87.65	1.01	NS	A *
	F	94.85	93.00	1.10	NS	B *
Val	IL	94.20	91.10	0.41	**	A **
	F	97.55	97.57	0.79	NS	B **
Met	IL	98.35	95.80	0.25	**	A NS
	F	98.15	96.67	0.43	NS	B NS
Ile	IL	95.38	93.03	0.45	*	A *
	F	97.73	96.67	0.46	NS	B ***
Leu	IL	96.58	96.10	0.37	NS	A NS
	F	97.88	97.10	0.48	NS	B NS
Tyr	IL	97.05	96.18	0.28	NS	A NS
	F	97.10	97.03	0.44	NS	B NS
Phe	IL	95.83	95.63	0.36	NS	A *
	F	97.65	96.83	0.52	NS	B NS
His	IL	96.10	93.20	0.31	***	A *
	F	98.08	96.87	0.42	NS	B ***
Lys	IL	96.43	57.07[5]	0.96	***	A *
	F	98.30	97.87	0.50	NS	B ***
Arg	IL	94.78	88.00	0.81	**	A NS
	F	96.23	94.43	0.71	NS	B **
Nit	IL	89.83	84.25	0.73	**	A ***
	F	97.70	95.37	0.28	**	B ***

Table 4.6 [1]1-Fluoro-2,4-dinitrobenzene reactive lysine. [2]Acid hydrolysis lysine. [3]Furosine detected in ileal digesta after acid hydrolysis, expressed in lysine units. [4]Regenerated lysine for the diet = 1.6 x F and for ileal digesta = 2.4 x F. [5]Actual lysine = AHL - RL. [6]The lysine attributed to being either free or N-terminally bound. [7]The proportion of the total lysine which is C-terminally or internally bound. For diet A n = 4. For diet BR n = 3; pig #3 was excluded due to abnormally high concentrations of ε-DF-L. **NA** = not applicable

Table 4.7 [1]Acid hydrolysis lysine. [3]Actual lysine. For diet A n = 4. For diet BR n = 3; pig #3 was excluded due to excessively high concentrations of ε-DF-L.

Table 4.8 [1](Ileal values/g Cr - Faecal values/g Cr)/(Ileal values/g Cr). For diet A n = 4. For diet BR n = 3, except for faecal values; pig #3 was excluded due to abnormally high concentrations of ε-DF-L.

Table 4.6 *Mean (± SE) FDNB reactive lysine, acid hydrolysis lysine, furosine, regenerated lysine and actual lysine in the diet and ileal digesta from pigs fed the non-heated diet (A) or heated diet (BR).*

	Compound (mmol/g Cr)						
	FDNB[1]	AHL[2]	F[3]	RL[4]	AL[5]	(AL-FDNB)[6]	(FDNB/AL)[7]
Diet A	40.9	38.9	-	-	38.9	NA	NA
Ileal	1.03 ± 0.10	1.51 ± 0.13	-	-	1.51 ± 0.13	0.48 ± 0.11	0.703 ± 0.066
Diet BR	19.2	26.0	3.81	6.10	19.9	NA	NA
Ileal	2.00 ± 0.10	12.0 ± 0.3	1.60 ± 0.17	3.85 ± 0.42	8.10 ± 0.5	6.10 ± 0.37	0.247 ± 0.003

Table 4.7 *Mean (± SE) ileal lysine digestibility coefficients (%) as determined from acid hydrolysed lysine (AHL) and actual lysine (AL) values from pigs fed the non-heated diet (A) or the heated diet (BR).*

	Lysine digestibilities (%)	
Diet	AHL[1]	AL[2]
A	96.3 ± 0.3	96.3 ± 0.3
BR	54.0 ± 1.3	59.3 ± 2.3

Table 4.8 *Mean (±SE) ileal and faecal digestibility coefficients (%) of ε-DF-L, total and residual radioactivity and the mean digestibility occurring in the large intestine for pigs fed the heated diet (BR).*

Source	ε-DF-L	Radioactivity	
		Total	Residual
Ileal	49.0 ± 5.9	69.5 ± 1.2	76.5 ± 5.0
Faecal	97.0 ± 0.3	93.7 ± 0.2	91.8 ± 0.3
Digestibility occurring in the large intestine[1]	95.0 ± 1.0	82.4 ± 2.5	58.1 ± 7.1

4.3.2 The pattern of digestion and absorption from the stomach to the terminal ileum

The sections of the small intestine which were isolated for removal of contents were of similar size (around 1,500 cm). There was only a small amount of material present in the first section with the amount gradually building-up towards the end of the small intestine. The contents of the first section were yellow in appearance, containing only trace amounts of chromium. The second and third sections were notably variable between individual animals in their contents. The amounts of dry matter found in the stomach and various sections of the small intestine are given in Table 4.9, along with single values (from pooled samples across pigs for each diet) for the amounts of chromium in the various sections and the marker retention times.

Of the dry matter ingested there appeared to be a greater recovery from stomach plus small intestine for diet A (57 %) than for diet BR (46 %), although this difference was not statistically significant. Pigs fed diet BR, however, tended to have ($P < 0.1$) less dry matter in their stomachs. The total amount of dry matter in the small intestine was higher ($P < 0.001$) for pigs given diet BR, with the values for each section of the small intestine (except section 1) also being higher for diet BR verses diet A. The inter-animal variability was high for dry matter recovery, for the first half of the small intestine.

The total recovery of chromium from the mouth to the end of the small intestine was high for both treatments being 108 % of that fed for diet A and 104 % for diet BR. The distribution of chromium in the gastro-intestinal sections differed between treatments with more chromium in the stomach than the small intestine for diet A but the converse for diet BR. This is also shown by the differences in the calculated retention times of chromium which are indicative of the digesta retention times. The contents from diet A remained in the stomach 79 min longer than the contents from diet BR, whereas the digesta in the small intestine from diet BR remained 86 min longer than the digesta from diet A. The overall retention times of digesta from the stomach to the terminal ileum of the small intestine were approximately the same.

The distribution of Maillard products in the contents and mucosal walls from the gastro-intestinal sections are given in Table 4.10. The recovery of ingested Maillard products in the gastro-intestinal lumen and mucosa was particularly high for ε-DF-L (101 %),

whereas the recoveries of total radioactivity (57 %) and residual radioactivity (41 %) were much lower.

The stomach mucosa contained very low (< 1 % of amount found in the chyme) amounts of Maillard products, whereas in the small intestinal mucosa the proportions were much higher, being between 7 and 10 % of that in the small intestinal contents. It is also notable that there was a higher proportion of the total radioactivity as ε-DF-L in the small intestinal mucosa (70 %) than the stomach mucosa (50 %). Furthermore, over the small intestine, the amounts of Maillard products in the mucosa as expressed as proportions of amounts in the contents were highest around the fourth section for ε-DF-L (11 %) and total radioactivity (16 %) but increased steadily till the sixth section for residual radioactivity (6.5 %).

The digestibilities of dry matter, amino acids, nitrogen and the Maillard products in different sections of the gastro-intestinal tract (from the stomach to the terminal ileum) were determined from digesta samples pooled across pigs (according to diet A or BR). Consequently, the data described in the following section (reported in Appendix 4.3) only indicate possible trends.

Values found for the third to the sixth intestinal section showed that the digestion of dry matter, amino acids (except for glycine and lysine) and nitrogen, for both diets, was virtually complete by the third intestinal section (approximately 80 % digested) and only increased to a small degree to the sixth section. Also, these data generally indicate that there was little difference between diets A and BR for the digestion of dry matter, amino acids (except for lysine) and nitrogen.

The digestibility of acid hydrolysed lysine for diet BR, decreased progressively from the third section on, and was considerably lower within each section than for diet A. The digestibility of actual lysine (corrected for the regenerated lysine from the hydrolysis of ε-DF-L) also decreased along the small intestine, but the digestibility of actual lysine was always higher than for acid hydrolysed lysine.

The digestion of Maillard products (ε-DF-L, total radioactivity and residual radioactivity) also decreased from the third intestinal section onwards, particularly for ε-DF-L as the digestibility actually became negative by the fourth intestinal section.

Table 4.9 [1]The chromium data is on samples pooled across pigs according to diet (A or BR). [2]Overall standard error. [3]Statistical level of significance: **NS** = not significant; **NS**$^{+}$ = ($P < 0.1$); * ($P < 0.05$); *** ($P < 0.001$). [4]Small intestinal section 6 includes the ileal digesta samples which were collected separately. **ND** = not determined.

It is of particular interest that concentrations (expressed relative to chromium) of ε-DF-L, total radioactivity, amino acids, nitrogen and dry matter from the sixth intestinal section were higher than the corresponding mean concentrations in the individual terminal ileal digesta samples (1.8 x, 1.5 x, 1.5 x, 1.3 x, and 1.1 x, respectively). A further point is that for the Maillard products, particularly ε-DF-L, there was a much higher difference than for the dry matter, amino acids and nitrogen.

Table 4.9 *Mean (± SE) dry matter contents, chromium content and chromium retention times in sections of the gastrointestinal tract from the stomach to the terminal ileum in pigs fed the non-heated diet (A) or the heated diet (BR).*

Region	Dry matter (DM) content (mg/g DM intake)				Chromium (Cr)[1] Content (mg/g Cr intake)		Retention time (min)	
	A	BR	(SE)[2]	Level of sig[3]	A	BR	A	BR
Stomach:	499.0	339.0	61.0	NS+	583.0	392.0	244.0	165.0
Small intestine:								
1	3.9	3.7	0.9	NS	ND	ND	ND	ND
2	7.4	11.0	3.0	NS	5.2	27.7	2.2	11.4
3	8.2	20.4	7.3	NS	49.4	88.1	20.7	36.9
4	8.5	15.4	2.8	NS	58.9	80.0	24.7	33.3
5	16.7	36.3	6.4	*	92.5	184.0	39.0	78.0
6[4]	27.4	31.7	2.1	NS	291.0	271.0	126.0	128.0
Total	72.1	118.5	12.4	***	497.0	650.8	212.6	287.6
TOTAL	571.1	457.5	33.0	NS	1080	1043	457	453

Table 4.10 [1]Residual radioactivity (total radioactivity - radioactivity attributed to ε-DF-L, 3.33×10^9 dpm/mol). [2]Small intestinal section 6 includes the ileal digesta samples which were collected separately. **ND** = not determined.

Table 4.10 *The total ε-DF-L, total radioactivity and residual radioactivity fed to pigs over 7 hourly feeds and the distribution in the contents (C) and mucosa.(M) in sections of the gastro-intestinal tract from the stomach to the terminal ileum in pigs fed the heated diet (BR).*

			Radioactivity (dpmx10^5)			
	ε-DF-L (mmol)		Total		Residual[1]	
Total units fed per day						
	12.9		1660		1230	
Gastro-intestinal region (total units recovered in the contents or mucosa)						
	(C)	(M)	(C)	(M)	(C)	(M)
Stomach:	3.80	0.020	460	1.39	333	1.30
Small intestine:						
1	ND	ND	ND	ND	ND	ND
2	0.14	0.065	9.9	2.02	5.1	0.00
3	1.38	0.132	76.5	5.03	30.5	0.65
4	1.04	0.162	55.3	6.23	20.3	0.61
5	2.99	0.265	171.0	12.80	71.3	4.00
6[2]	2.91	0.140	136.0	7.18	39.0	2.53
Total	8.46	0.764	448.7	33.3	166.2	7.79
TOTAL	12.26	0.784	908.7	34.7	499.2	9.09

4.3.3 Maillard product concentrations in the portal and jugular serum

The concentrations of ε-DF-L and radioactivity in the portal and jugular serum from pigs fed diet BR, one hour after the seventh hourly feed, are reported in Table 4.11. The ε-DF-L concentrations were determined on the acid hydrolysates of serum supernatants after precipitation of proteins and peptides with 10 % TCA; the latter was necessary as large amounts had to be loaded to detect the small amounts of furosine present.

Maillard products were detected in both the portal and jugular serum. The proportions of the total radioactivity in the serum as ε-DF-L changed slightly between the portal (42 %) and jugular (35 %) serum. No furosine was detected in the serum hydrolysates of the pigs fed diet A.

Table 4.11 *Concentrations of ε-DF-L and total radioactivity in the portal and jugular serum of pigs fed the heated diet (BR).*

ε-DF-L (nmol/ml serum)		Total radioactivity (dpm/ml serum)	
Portal	Jugular	Portal	Jugular
23.9 ± 1.3	11.4 ± 0.4	191 ± 10	108 ± 2

4.3.4 The molecular weight distribution of compounds (amino acids, peptides and Maillard products) in ileal digesta

In this section the distribution of amino acids, peptides and Maillard products in the ileal digesta samples (pooled according to the diet that the pigs received, either diet A or diet BR), were determined using gel chromatography with a calibrated Sephadex G-15 column. The molecular weight calibration curve of the prepared column (void volume approximately 120 ml) is given in Appendix 4.4. Processing of the ileal digesta and the chromatography technique have been described in detail previously (refer methods section 4.2.5).

The pooled freeze-dried ileal digesta samples were dissolved in acetic acid (300 mg/5 ml of 0.2 M acetic acid), but did not completely solublize, with some insoluble residue remaining after centrifugation. The supernatants from both diets were cloudy in appearance.

The elution profiles for the supernatants (3 ml loaded), at absorbances of 280 and 420 nm (Figure 4.3 I and 4.3 II respectively) differed considerably between diets. The cloudy material present in the supernatants from both diets, probably eluted at the void volume, as the eluates in this region were also cloudy and high absorbances were recorded. Apart from the values around the void volume, absorbance values for diet BR were generally much higher than for diet A. The largest difference was noted about fraction 21.

The distribution of ninhydrin positive compounds (Figure 4.3 III) also showed major differences between the diets. The greatest absorbance for the ninhydrin positive compounds for diet A corresponded to a 220 molecular weight region, whereas the greatest absorbance for diet BR was around a molecular weight 430, with much lower absorbances in the 220 molecular weight region. Most of the radioactivity for diet BR (Figure 4.3 IV) was also present in the ninhydrin positive 430 molecular weight region.

The fractions for both diets were divided into five regions corresponding to 1500, 790, 430, 220 and below 200 molecular weight (refer Figure 4.3). The first three regions were considered to contain peptides, whereas the last two regions were considered to mainly contain free amino acids. The respective fractions in each region were pooled according to diet and analysed for amino acids, ε-DF-L and radioactivity. These values were expressed per g of chromium in the original ileal digesta and are given in Table 4.12.

On average approximately 80 % of the amino acids present in the ileal digesta of both diets, was recovered in these five regions. The recoveries of individual amino acids varied quite widely (from as low as 50 % for glycine to as high as 95 % for serine), but the values were similar for the two diets. The proportions of amino acids, "peptide-bound" or present in the "free" form, differed for the two diets. A much higher percentage of the total amino acids recovered were present in the "peptide-bound" regions for diet BR than for diet A (82 and 67 % respectively). The increased quantity of amino acids in these regions (1, 2 and 3) for diet BR (an additional 12.6 mmol/g Cr compared to diet A), easily accounts for the higher amino acid content of the ileal digesta of these animals compared to the amount present in diet A (55.7 v 45.9). Approximately half of the "peptide-bound" amino acids from diet BR were found in region 3, whereas for diet A, the total amino acid contents of each of the three "peptide-bound" regions, were similar. Region 3, from diet BR, also contained nearly ten times as much actual lysine and arginine, and at least twice as much proline, valine and methionine, compared to the amount present in the same region of diet A. In addition, actual lysine, methionine and also histidine were twice as abundant in regions 1 and 2 for diet BR than for diet A.

All of the amino acids which were more abundant in the "peptide-bound" regions of diet BR (except for lysine), were present in lower amounts in the "free" regions 4 and 5, as

were most other amino acids, compared to diet A. The amount of actual lysine in these regions was, however, similar for the two diets.

Of the total radioactivity and ε-DF-L present in the ileal digesta, approximately 88 % was recovered in the combined fractions of the five regions. Maillard products were mainly located in region 3, which contained 70 % of the recovered total radioactivity and 85 % of the ε-DF-L. The ε-DF-L made up the major part (72 % of the radioactivity) of the Maillard products present in this region. The low molecular weight regions 4 and 5 contained the remaining 14 % of the radioactivity recovered, but no ε-DF-L was detected in these regions.

Figure 4.3 Ileal digesta from pigs fed either the non-heated diet A (◇) or the heated diet BR (▩) showed different absorbance elution profiles at (I) 280 nm and (II) 420 nm. From these distributions, and those on the following page, fractions were pooled into five regions. Three "peptide-bound" regions: region 1 (fractions 11 - 14, ≥ 1500 MW), region 2 (fractions 15 - 19, 790 MW), region 3 (fractions 20 - 25, 430 MW) and two "free: regions: region 4 (fractions 26 - 29, 220 MW) and region 5 (fractions 30 - 50, below 200 MW). The calibration curve is reported in Appendix 4.4, with the void volume (120 ml) at fraction 14 (1,500 MW) and urea eluting at fraction 40 (60 MW). The composition and distribution of radioactivity, ε-DF-L and individual amino acids in these pooled regions are reported in Table 4.12.

Figure 4.3 *Sephadex G-15 chromatography of ileal digesta from pigs fed either the non-heated diet (A) or the heated diet (BR): (I) Absorbance at 280 nm; (II) Absorbance at 420 nm; (III) Absorbance at 570 nm after reaction with ninhydrin; and (IV) Radioactivity due to* ^{14}C.

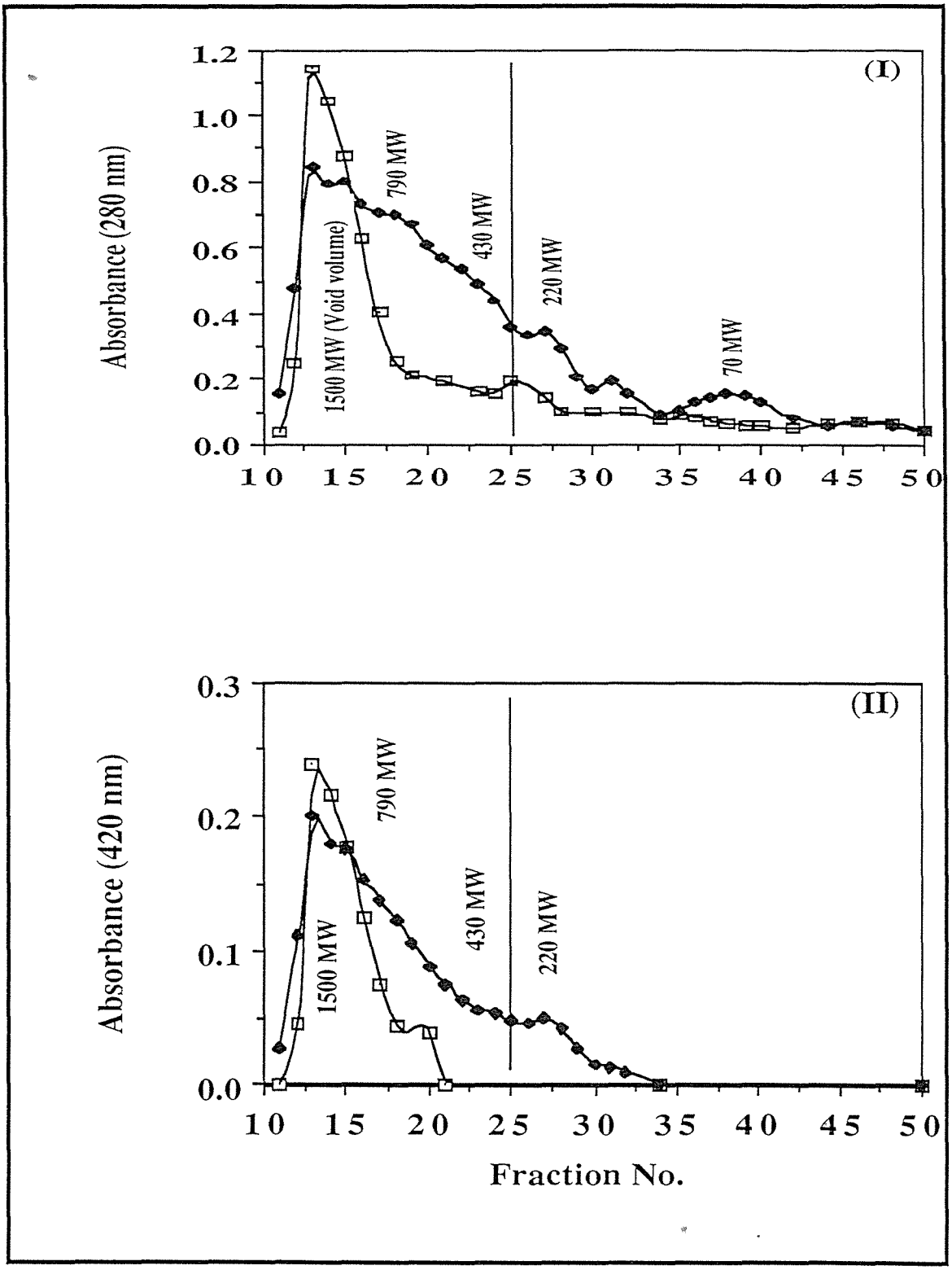


Figure 4.3 (continued) Non-heated diet A (◇) and heated diet BR (■). (III) The distribution of ninhydrin positive compounds were different between treatments. (IV) The ^{14}C radioactivity distribution of treatment BR associated most of the radioactivity in the "peptide-bound" region from fractions (20 - 25), which also corresponded with the majority of ninhydrin positive compounds.

Figure 4.3 *(Continued).*

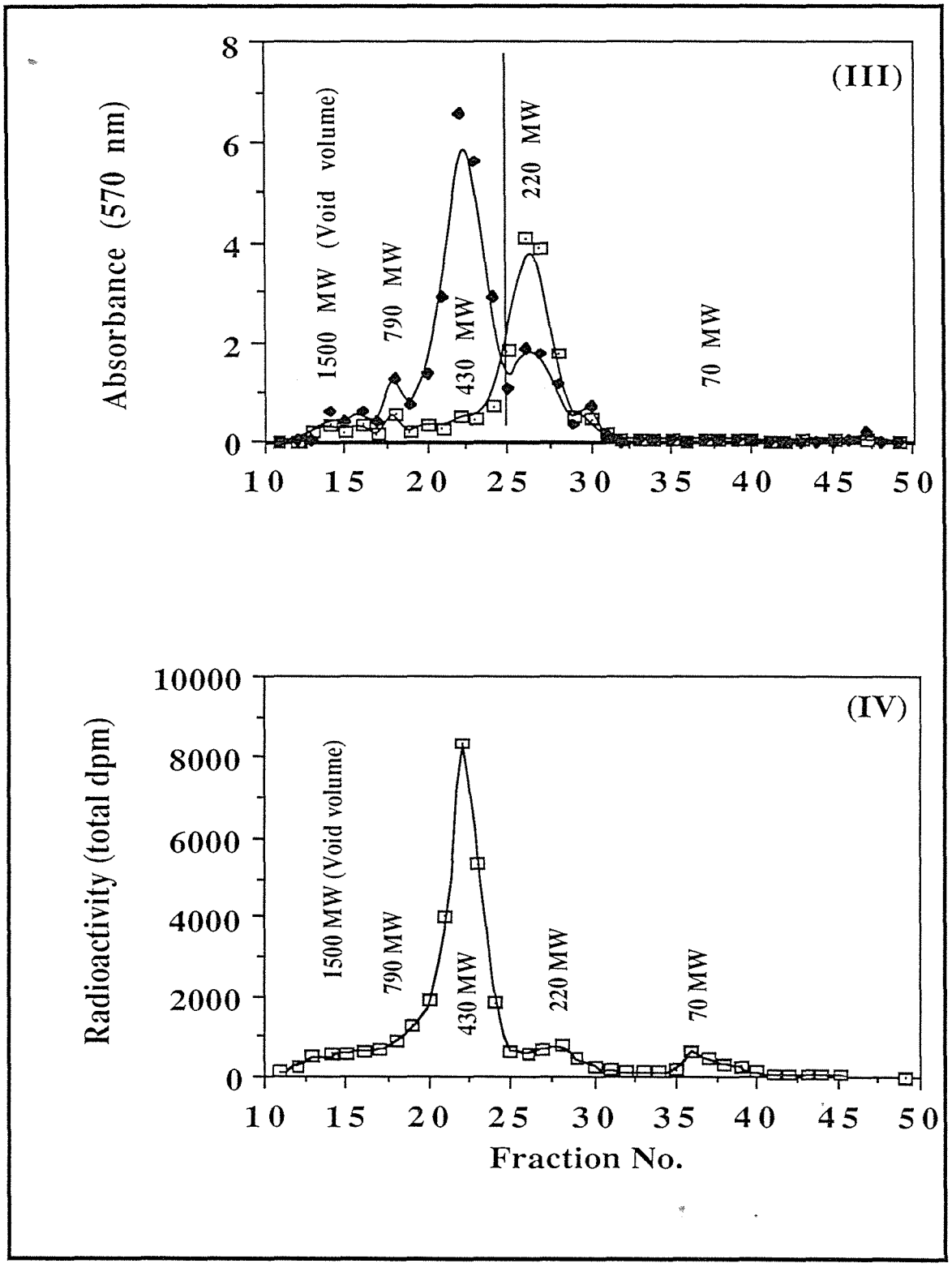


Table 4.12 The peptide bound and free regional divisions are shown in Figure 4.3. [1]**Asp** = aspartic acid, **Thr** = threonine, **Ser** = serine, **Glu** = glutamic acid, **Pro** = proline, **Gly** = glycine, **Ala** = alanine, **Val** = valine, **Met** = methionine, **Ile** = isoleucine, **Leu** = leucine, **Tyr** = tyrosine, **Phe** = phenylalanine, **His** = histidine, **Lys** = lysine, and **Arg** = arginine. [2]Residual radioactivity in lysine units was calculated from the specific radioactivity of residual radioactivity (11 $x10^9$ dpm/mol of reacted lysine) after subtracting the radioactivity attributed to ε-DF-L (3.33$x10^9$ dpm/mol) from the total radioactivity - 3.3.3. [3]Actual lysine estimates, determined by subtracting regenerated lysine (from the hydrolysis of ε-DF-L) from acid hydrolysis lysine. The majority of the Maillard products were located in region 3 (a 430 MW peptide region), which also contained the largest increases in the amino acid content from diet BR compared to diet A.

Table 4.12 *The amino acid and Maillard product composition and distribution in the pooled fractions after Sephadex G-15 chromatography of ileal digesta from pigs fed either the non-heated diet (A) or the heated diet (BR).*

	"Peptide-bound" regions			"Free" regions		Total	Total in ileal digesta
	1	2	3	4	5		
NONHEATED DIET (A):							
Amino acids[1] (mmol/g Cr)							
Asp	0.59	0.54	1.76	1.01	0.12	4.03	4.21
Thr	1.39	0.51	0.57	0.31	0.03	2.81	3.73
Ser	0.98	0.82	0.87	0.67	0.07	3.40	3.69
Glu	0.82	0.90	2.45	2.89	0.50	7.56	7.92
Pro	1.41	0.77	0.70	0.40	0.25	3.53	4.00
Gly	0.55	0.43	1.00	2.45	0.15	4.57	8.86
Ala	0.63	0.46	0.66	0.48	0.03	2.25	2.54
Val	0.53	0.44	0.50	0.42	0.04	1.93	2.30
Met	0.03	0.04	0.06	0.09	0.02	0.34	0.29
Ile	0.25	0.23	0.20	0.34	0.02	1.05	1.30
Leu	0.25	0.23	0.18	0.43	0.05	1.14	1.74
Tyr	0.08	0.11	0.04	0.03	0.19	0.50	0.65
Phe	0.10	0.09	0.05	0.03	0.23	0.49	0.93
His	0.08	0.10	0.21	0.04	0.01	0.44	0.53
Lys	0.13	0.25	0.54	0.13	0.03	1.08	1.39
Arg	0.12	0.10	0.13	0.10	0.24	0.69	0.84
Total	7.94	6.12	9.92	9.84	1.99	35.81	44.92
HEATED DIET (BR):							
Maillard products:							
Radioactivity (dpmx10^6/g Cr)	1.98	5.93	34.61	3.96	2.97	49.45	56.32
ε-DF-L (mmol/g Cr)	0.28	1.01	7.45	0	0	8.74	9.70
Residual radioactivity in lysine units (mmol/g Cr)[2]	0.10	0.25	0.86	0.35	0.26	1.82	2.11
Amino acids[1] (mmol/g Cr):							
Asp	0.77	0.78	1.74	0.51	0.11	3.91	4.24
Thr	1.38	0.94	0.71	0.18	0.03	3.25	3.70
Ser	1.09	1.18	1.05	0.40	0.09	3.80	3.92
Glu	1.29	1.34	2.38	1.71	0.40	7.12	7.47
Pro	1.25	1.09	1.49	0.22	0.12	4.17	4.91
Gly	0.55	0.44	0.69	1.71	0.16	3.54	8.45
Ala	0.60	0.42	0.77	0.28	0.05	2.11	2.93
Val	0.64	0.64	1.44	0.27	0.07	3.06	3.77
Met	0.10	0.11	0.14	0.06	0.02	0.43	0.58
Ile	0.47	0.38	0.24	0.31	0.03	1.44	2.00
Leu	0.27	0.24	0.18	0.35	0.07	1.10	1.99
Tyr	0.12	0.19	0.04	0.03	0.21	0.60	1.06
Phe	0.13	0.13	0.06	0.04	0.25	0.60	0.95
His	0.18	0.27	0.31	0.03	0.01	0.80	0.91
Lys[3]	0.32	0.51	5.96	0.08	0.08	6.95	8.08
Arg	0.10	0.13	1.18	0.22	0.02	1.65	1.78
Total	9.26	8.77	18.38	6.39	1.73	44.53	56.73

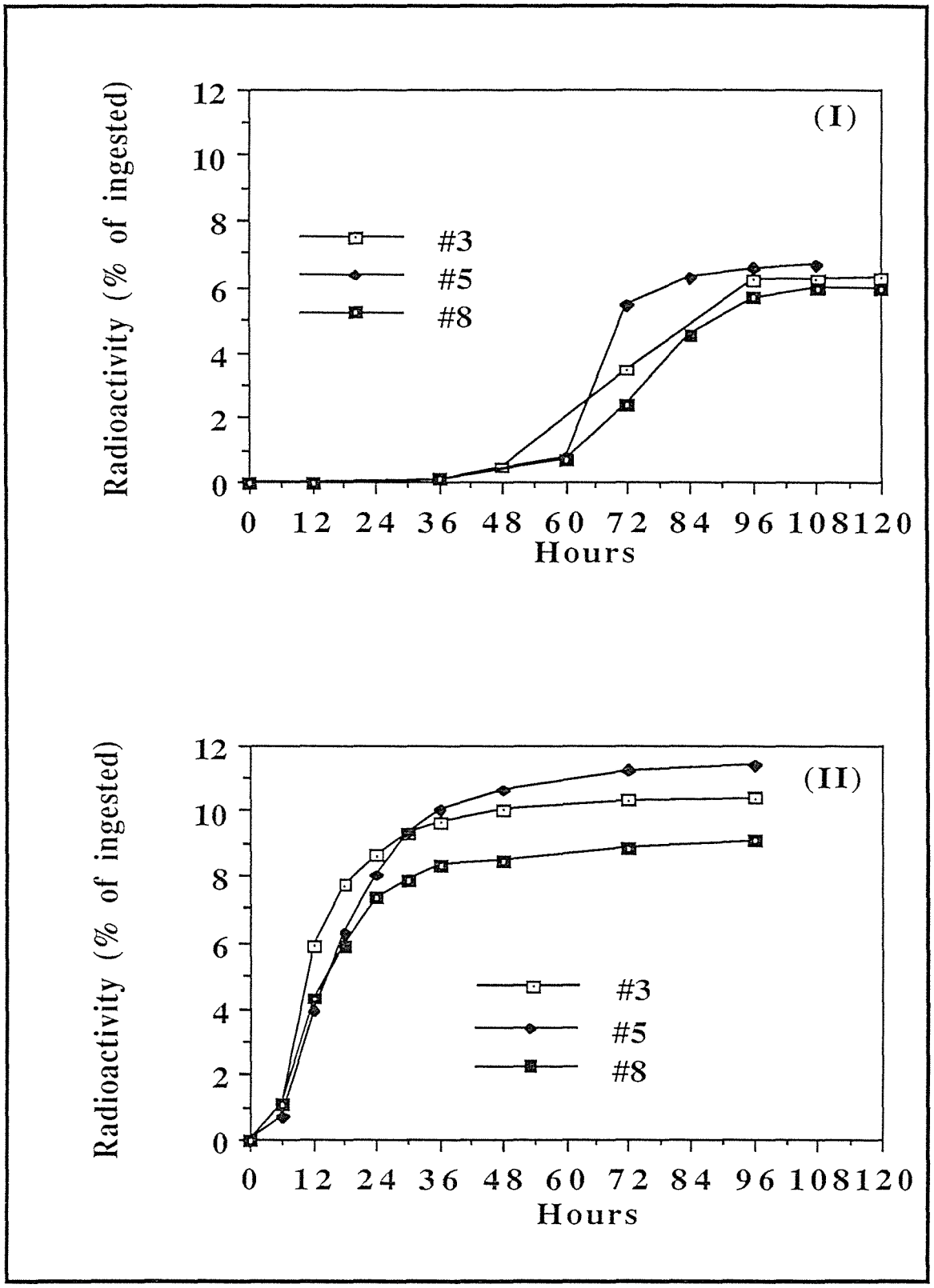
4.3.5 The pattern of ^{14}C-radioactivity excretion in faeces and urine from pigs fed a single meal of the radioactively labelled heated diet BR

A pulse (radioactively labelled heated diet BR) chase (heated diet B) feeding regime was undertaken, to determine the pattern of radioactivity excretion in the faeces and urine, from a single meal of diet BR. The faecal and urinary excretions of ^{14}C-radioactivity, expressed as cumulative percentages of ingested radioactivity, are graphed in Figure 4.4 (I & II respectively).

Radioactivity first appeared in the faeces 48 - 60 hours after feeding, and no further radioactivity was excreted after 84 - 96 hours. Small differences were noted between pigs in their defecation times. However, the total excretions were similar, stabilizing on average to about 6.30 ± 0.17 ($x \pm SE$; $n = 3$) % of the ingested radioactivity.

Radioactivity first appear in the urine after 6 hours, and little additional radioactivity was excreted after 36 - 48 hours following ingestion of the radioactive meal. The excretion of radioactivity in the urine was virtually completed before the faecal excretion had begun. The total excretion of radioactivity in the urine was almost twice that in the faeces. The mean ($x \pm SE$; $n = 3$) total excretion of radioactivity in the urine was 10.3 ± 0.5 % of that ingested.

Figure 4.4 *Cumulative excretion (as a % of that ingested) of ^{14}C-radioactivity in the: (I) faeces; and (II) urine, after ingestion of a single meal of radioactively labelled heated diet (BR).*



4.3.6 Urinary excretion of nitrogen, amino acids and Maillard products

Complete urinary collections were made over the four day collection period and there was no cross contamination with faecal material.

The amino acid chromatograms of urinary samples (after acid hydrolysis) were complicated, as they contained many ninhydrin positive compounds. The chromatograms were not totally resolvable, with double peaks and shoulders occurring. Therefore, the following results (Table 4.13) should be considered as only approximating the total amino acid (from peptides and free molecules) content.

The excretion of amino acids and nitrogen in urine were expressed in two ways. The first method expressed excretion as a proportion of the amount of the amino acid apparently absorbed from the gastro-intestinal tract. The amount of each amino acid absorbed was determined by multiplying dietary intake (g per $kg^{0.75}$ per day) by the respective apparent ileal digestibility coefficient reported in Table 4.5. The second method expressed the amount excreted per gram of urinary creatinine.

Regardless of the method of expressing amino acid excretion, there was little effect of diet. Only the values for acid hydrolysed lysine were significantly ($P < 0.01$) different between the diets, with a much higher proportion of the absorbed lysine being excreted in the urine for pigs fed diet B compared to those on diet A. Excluding lysine and glycine, the excretion of all other amino acids measured was always less than 3 % of the absorbed amount. The value for glycine was high for both treatments, however, the ileal digestibility coefficients of this amino acid were low and variable. There was no significant effect of diet on the urinary excretion of total nitrogen.

The urinary excretion of ε-DF-L, total radioactivity, residual radioactivity and actual lysine for pigs fed the heated diets (B and BR), expressed either as a proportion of the amount of each material ingested, or the amount apparently absorbed at the terminal ileum, are given in Table 4.14.

The amount of actual lysine excreted (accounting for the lysine regenerated from the hydrolysis of ε-DF-L) for diet B (0.56 %), expressed as a percentage of actual lysine absorbed, was much lower than the corresponding acid hydrolysis values, but was

similar to that determined for diet A (0.74 %). Thus, the high value for the excretion of acid hydrolysed lysine (reported in Table 4.13), for pigs fed diet B (6.78 %), must be mainly due to lysine regenerated from the excretion of ε-DF-L. Furthermore, expression of actual lysine excreted per g creatinine indicated that the mean ($x \pm SE$; $n = 4$) amount in the urine for pigs fed diet B (0.085) was significantly lower ($P < 0.05$) than for pigs fed diet A (0.139).

The radioactivity attributable to ε-DF-L accounted for a lower proportion, 27.8 ± 2.8 ($x \pm$ SE; $n = 3$) %, of the total radioactivity than did residual radioactivity. The values for ε-DF-L given in Table 4.14, show that about 21 % of that absorbed was excreted in the urine, but the percentage of ε-DF-L excreted from that ingested was half of this (11 vs 21 %). However, the values based on absorption were calculated assuming that half of the ε-DF-L in the ileal digesta was protein bound, with the other half being in the free form. If all the ε-DF-L is assumed to be in only one of the two possible states (bound or free), then the percentage excreted (of that absorbed) varies from 17 to 36 %. By the same reasoning the excretion of residual radioactivity could also range from 22 to 29 %. Thus, although the calculated values vary considerably depending on the assumption on which the calculations are based, it is nevertheless clear that a substantial proportion of apparently absorbed Maillard products (ε-DF-L and other compounds) are not excreted in the urine.

Table 4.13 [1] $\frac{\text{Daily excretion/kg}^{0.75}}{\text{(Daily intake/kg}^{0.75}\text{ x ileal digestibility coefficient)}} \times \frac{100}{1}$

Ileal digestibility coefficients are reported in Table 4.5.

[2] **Asp** = aspartic acid, **Thr** = threonine, **Ser** = serine, **Glu** = glutamic acid, **Pro** = proline, **Gly** = glycine, **Ala** = alanine, **Val** = valine, **Met** = methionine, **Ile** = isoleucine, **Leu** = leucine, **Tyr** = tyrosine, **Phe** = phenylalanine, **His** = histidine, **Lys** = lysine, **Arg** = arginine and **Nit** = nitrogen. [3]Overall standard error [4]Statistical significance: **NS** = not significant; **NS**$^{+}$ ($P < 0.1$); ** ($P < 0.01$) and *** ($P < 0.001$).

Table 4.14. [1]Residual radioactivity (total radioactivity - radioactivity attributed to ε-DF-L, 3.33×10^9 dpm/mol - refer 3.3.3) [2]Actual lysine estimates, determined by subtracting regenerated lysine, from the hydrolysis of ε-DF-L, from acid hydrolysed lysine.

[3] $\frac{\text{Daily excretion/kg}^{0.75}}{\text{Daily intake/kg}^{0.75}} \times \frac{100}{1}$

[4] $\frac{\text{Daily excretion/kg}^{0.75}}{\text{(Daily intake/kg}^{0.75}\text{ x ileal digestibility coefficient)}} \times \frac{100}{1}$

Ileal digestibility coefficeints are reported in Table 4.5.

[5]Expressed in lysine units (11×10^9 dpm/mol of reacted lysine).

Table 4.13 *Mean urinary excretion of amino acids (after acid hydrolysis) and nitrogen, expressed either as a proportion of the amount of each amino acid or nitrogen apparently absorbed at the terminal ileum, or per unit urinary creatinine, for pigs fed the non-heated diet (A) or the heated diet (B).*

	Urinary excretion							
	% of that absorbed[1]				g/g creatinine			
Amino acid[2]	Diet (A)	Diet (B)	SE[3]	Sig[4]	Diet (A)	Diet (B)	SE	Sig
Asp	2.06	1.79	0.28	NS	0.301	0.262	0.027	NS
Thr	1.46	1.42	0.25	NS	0.121	0.107	0.014	NS
Ser	1.13	1.34	0.19	NS	0.124	0.112	0.011	NS
Glu	1.35	0.98	0.30	NS	0.662	0.467	0.128	NS
Pro	0.96	1.16	0.22	NS	0.370	0.407	0.060	NS
Gly	19.31	26.01	3.10	NS+	0.548	0.573	0.054	NS
Ala	2.08	2.18	0.26	NS	0.135	0.133	0.012	NS
Val	0.70	0.90	0.10	NS+	0.100	0.127	0.010	NS+
Met	1.17	1.11	0.06	NS	0.081	0.062	0.004	NS
Ile	0.40	0.45	0.07	NS	0.050	0.055	0.006	NS
Leu	0.47	0.44	0.07	NS	0.097	0.097	0.009	NS
Tyr	0.50	0.65	0.07	NS+	0.073	0.083	0.008	NS
Phe	0.46	0.55	0.05	NS	0.662	0.769	0.128	NS
His	0.96	1.13	0.11	NS	0.065	0.071	0.005	NS
Lys	0.74	6.78	1.05	**	0.139	0.615	0.031	***
Arg	1.61	1.65	0.18	NS	0.148	0.119	0.012	NS±
Nit	28.22	32.26	1.16	NS	9.580	10.171	0.285	NS

Table 4.14 *Mean (± SE) urinary excretion of ε-DF-L, radioactivity and actual lysine, expressed as a percentage of ingested, or absorbed amounts for pigs fed the heated diets (B and BR).*

		Radioactivity		
	ε-DF-L	Total	Residual[1]	Actual lysine[2]
% of Ingested[3]	10.8 ± 0.8	10.3 ± 0.5	20.1 ± 1.7	0.44 ± 0.03
% of Absorbed[4]	21.2 ± 2.5	16.5 ± 1.5	25.5 ± 1.4	0.56 ± 0.04

4.3.7 The molecular weight distribution of compounds (amino acids, peptides and Maillard products) in urine

In this section the distribution of amino acids, peptides and Maillard products in urine samples (pooled across pigs according to the diet that the pigs received, either diet A or diet B), were examined by gel chromatography using a calibrated Sephadex G-15 column.

The values obtained for radioactivity in the urine for pigs on diet B were the result of only one meal of radioactively labelled heated diet (BR) fed at the beginning of the collection period. Therefore, there would have been a dilution from the urine produced after the consumption of the non-radioactive heated diet (B). This is not the case for ε-DF-L in the urine, as it was in approximately the same proportions in both of the heated diets (refer Chapter 3). In order to relate the radioactivity excreted to that expected if the radioactively labelled heated diet (BR) had been constantly fed, the following calculations were made. These calculations were based on the mean values obtained for the 3 pigs which received the single meal of diet BR, followed by diet B:

On average, 1.62×10^7 dpm were excreted in the urine over the 4 days of collection after feeding one meal of diet BR, which on average contained 1.57×10^8 dpm. Since two meals are given per day, 3.24×10^7 dpm would be expected to be excreted per day if diet BR was fed constantly. On average 1,171 mls of urine were voided per pig per day, and therefore the radioactivity expected would be 1.56×10^4 dpm/ml. However, with only one meal of diet BR, the actual radioactivity estimated was 0.346×10^4 dpm/ml. Therefore, to convert the measured radioactivity excretion values to those which would occur on constant feeding, the values were multiplied by 4.5 ($1.56 \times 10^4 / 0.346 \times 10^4$). This permitted an estimate to be made of the radioactivity associated with ε-DF-L, and hence, of the residual radioactivity.

Sephadex G-15 chromatography

The urine was chromatographed on the same column as that used to examine the ileal digesta with the same calibration curve being used (refer Appendix 4.4). The processing of the urine samples and the chromatography technique has been previously described in detail (refer methods section 4.2.5).

The pooled, freeze dried urine samples almost completely dissolved in the 0.2 M acetic acid (approximately 0.4 g/5 ml of 0.2 M acetic acid). However, there was a small amount of insoluble residue after centrifuging the suspensions. It was also noticed on loading the supernatants (3 ml loaded) to the column, that some brown material adhered to and discoloured the top portion of the column. This was particularly so for the more darker coloured urine obtained for pigs on diet B.

The elution profiles at absorbances of 280 and 420 nm (Figure 4.5 I and 4.5 II, respectively), differed considerably between diets. This was particularly the case between fractions 10 - 25, with much higher absorbance values for diet B. In particular, there were huge absorbance differences at 420 nm, with diet A having virtually no absorbance at this wavelength. For both diets, most of the ninhydrin positive compounds (Figure 4.5 III) eluted in fractions corresponding to a 175 molecular weight region, with diet A having slightly less ninhydrin positive material in this region.

Most of the radioactivity for diet B (Figure 4.5 IV) was concentrated in the fractions corresponding to a 715 molecular weight. These fractions also gave high absorbance values at 420 nm (refer Figure 4.6 II), where brown pigments are known to absorb, suggesting the presence of Maillard browned pigments. The amount of ninhydrin positive compounds in these fractions was low. The remaining radioactivity was spread over the fractions corresponding to compounds with molecular weights less than 290, in three distinct peaks. The last peak (fractions 36 - 42) coincides with the elution of urea (see calibration curve, Appendix 4.4).

The fractions from both diets were divided into four regions corresponding to molecular weights of 1500, 715, 430 and below 290, as shown on Figure 4.5. The first three regions were considered to peptides and the last region free amino acids. The fractions in each region were pooled and analysed for amino acids, ε-DF-L and radioactivity. The values were converted to total amounts excreted in the urine per $kg^{0.75}$ per day and are given in Table 4.15.

Unfortunately, low recoveries of the amino acids were obtained after chromatography. Overall only 44 % of the urinary amino acids for diet A and 41 % for diet B were recovered after chromatography. Individual amino acid recoveries ranged from 25 % for

proline to 68 % for tyrosine. Recoveries were similarly low for both diets. Results may have been influenced by these low recoveries. In the "peptide-bound" region 2 of diet B, there was at least twice as much proline, valine and methionine compared to diet A. In the "free" region 4, arginine, glutamic acid, actual lysine, phenylalanine and histidine were lower for diet B compared to diet A.

The second region also contained virtually all the ε-DF-L, and all the acid hydrolysed lysine appeared to have been regenerated from ε-DF-L for diet B, resulting in apparently no actual lysine. It is also of note that the amount of ε-DF-L in this region was much greater that the total quantity of amino acids estimated in this region. The ε-DF-L present accounted for a higher proportion (57 %) of the total radioactivity than the residual radioactivity. Calculation of the radioactivity not associated with ε-DF-L (i.e. residual radioactivity), indicated that other Maillard products were also probably present in the "peptide-bound" region 2. Most of the remaining radioactivity (33 %) was present in the "free" region 4, but no ε-DF-L was detected in this region. Unlike the recoveries of amino acids, the overall recovery of radioactivity from that present in the original urine, was quite reasonable (77 %), as was the recovery of ε-DF-L (81 %).

Figure 4.5 Urine from pigs fed either the non-heated diet A (□) or the heated diet (◆) have different absorbance elution profiles at (I) 280 nm and (II) 420 nm. From these distributions, and those on the following page, fractions were pooled into four regions. Three "peptide-bound" regions: region 1 (fractions 11 - 15, ≥ 1500 MW), region 2 (fractions 16 - 20, 715 MW), region 3 (fractions 21 - 25, 430 MW) and one "free region: region 4 (fractions 26 - 50, ≤ 290 MW). The calibration curve is reported in Appendix 4.4, with the void volume (120 ml) being around fraction 14 (1500 MW) and urea eluting around fraction 40 (60 MW). The composition and distribution of radioactivity, ε-DF-L and individual amino acids in these pooled regions are reported in Table 4.15.

Figure 4.5 *Sephadex G-15 chromatography of urine from pigs fed either the non-heated diet (A) or the heated diet (B): (I) Absorbance at 280 nm; (II) Absorbance at 420 nm; (III) Absorbance at 570 nm after reaction with ninhydrin; and (IV) Radioactivity due to ^{14}C.*

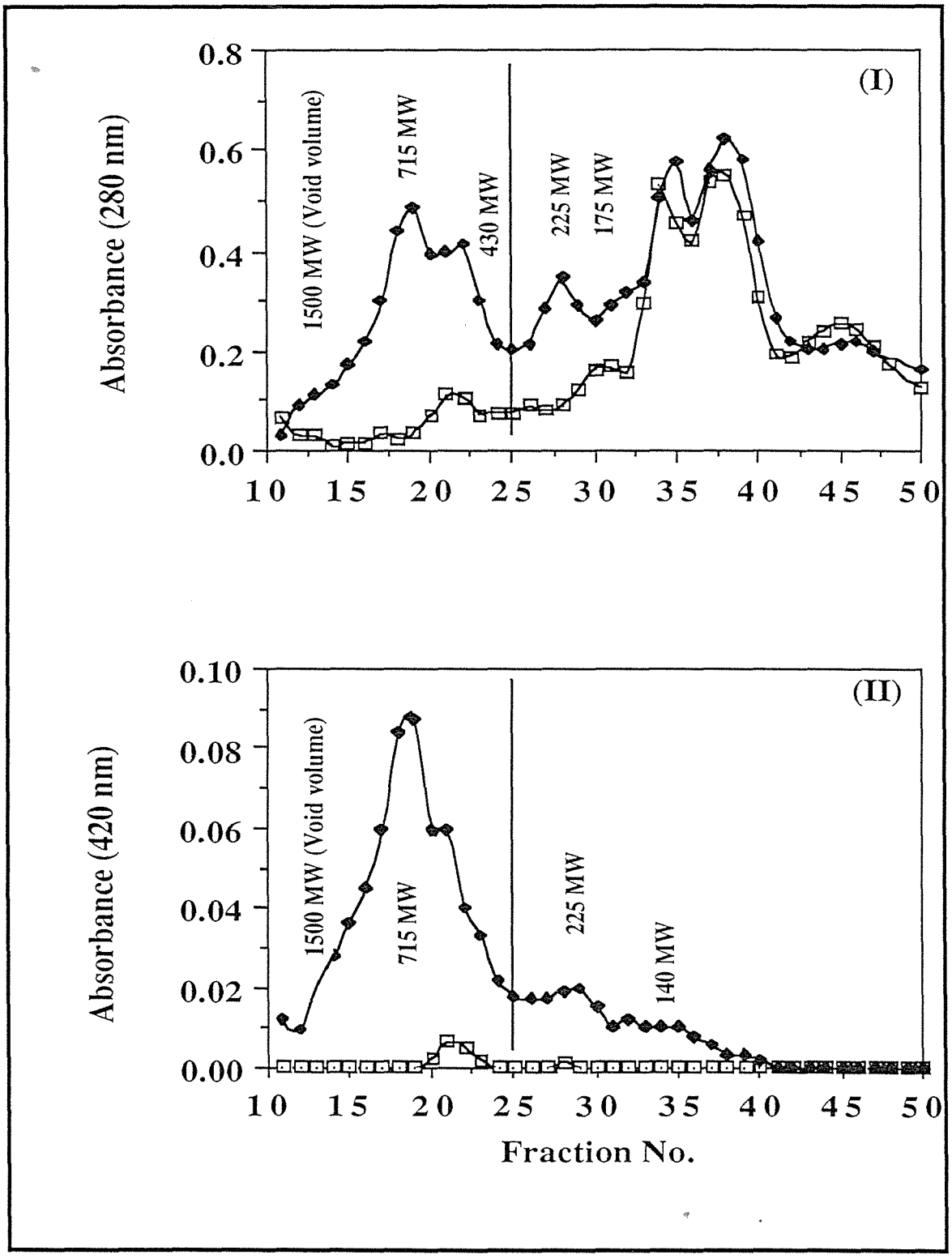


Figure 4.5 (continued) Non-heated diet A (□) and the heated diet B (◆). **(III)** The distribution of ninhydrin positive compounds are virtually the same between treatments. **(IV)** The radioactivity distribution of treatment BR associated most of the radioactivity in the "peptide-bound" region from fractions (17 - 20).

Figure 4.5 *(Continued).*

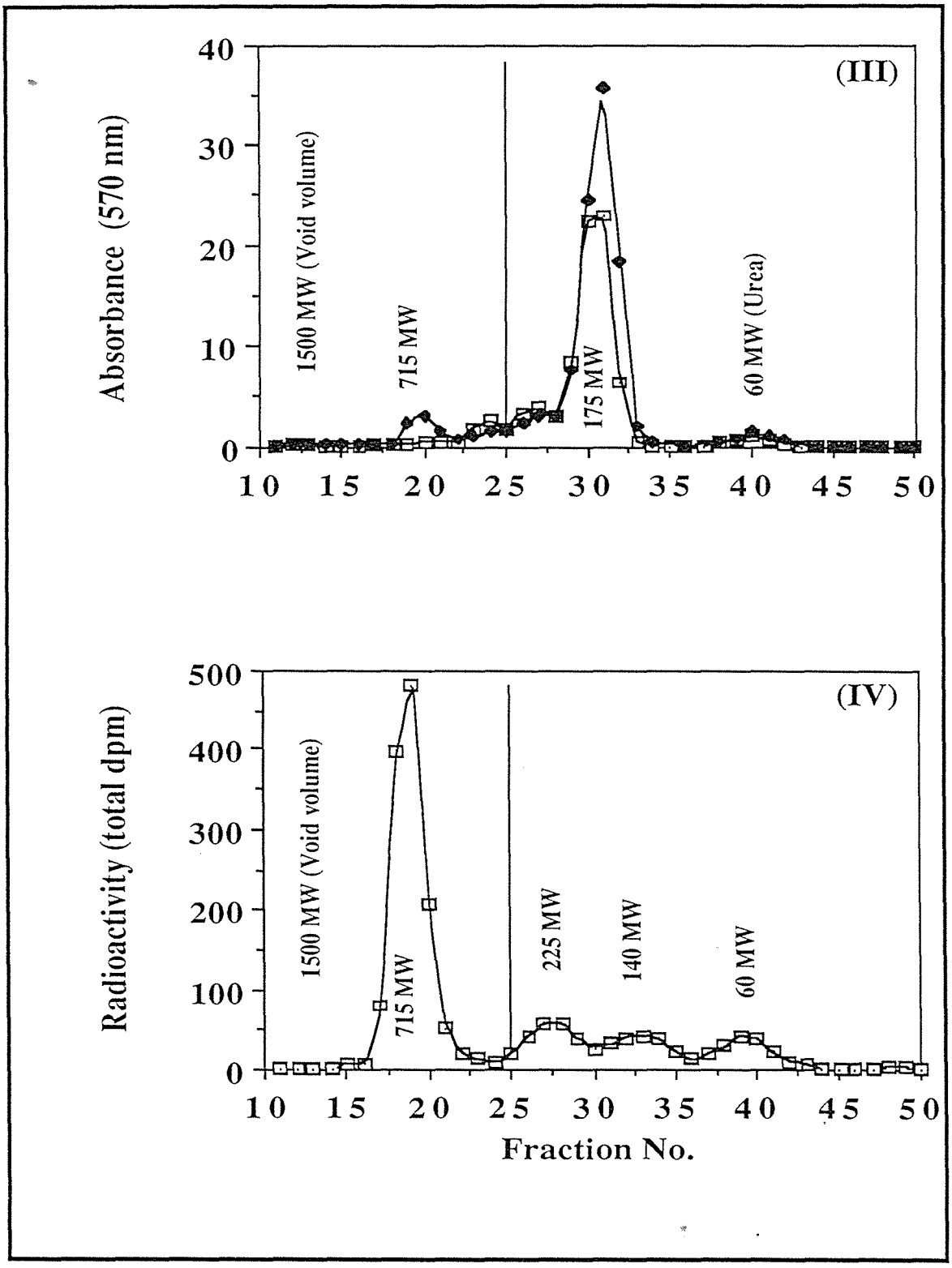


Table 4.15 The regional divisions are shown in Figure 4.5. [1] **Asp** = aspartic acid, **Thr** = threonine, **Ser** = serine, **Glu** = glutamic acid, **Pro** = proline, **Gly** = glycine, **Ala** = alanine, **Val** = valine, **Met** = methionine, **Ile** = isoleucine, **Leu** = leucine, **Tyr** = tyrosine, **Phe** = phenylalanine, **His** = histidine, **Lys** = lysine, and **Arg** = arginine. [2]Residual radioactivity in lysine units was calculated from the specific radioactivity of residual radioactivity (11 $x10^9$ dpm/mol of reacted lysine) after subtracting the radioactivity attributed to ε-DF-L ($3.33x10^9$ dpm/mol) from the total radioactivity - refer Chapter 3, 3.3.3. [3]Actual lysine estimates, determined by subtracting regenerated lysine (from the hydrolysis of ε-DF-L) from acid hydrolysis lysine. The majority of the Maillard products were located in region 2 (a 715 MW peptide region). This region also contained the greatest difference in the amino acid content between diets.

Table 4.15 *The amino acid and Maillard product composition and distribution in the pooled fractions from Sephadex G-15 chromatography of urine collected from pigs fed either the non-heated diet (A) or the heated diet (BR).*

	"Peptide-bound" regions			"Free" region	Total	Total in urine
	1	2	3	4		
NON-HEATED DIET (A):						
Amino acids[1] (μmol $(kg^{0.75})^{-1}$ day^{-1}):						
Asp	26.2	13.7	7.0	27.4	74.3	150.0
Thr	12.4	4.9	4.1	10.0	31.4	67.7
Ser	15.8	6.5	4.9	11.9	39.1	78.8
Glu	30.7	14.9	8.0	102.1	155.7	293.0
Pro	26.0	6.1	11.3	17.0	60.4	214.0
Gly	79.5	26.6	13.4	68.6	188.1	476.0
Ala	23.9	6.4	3.7	13.8	47.8	99.8
Val	9.9	4.4	2.3	9.0	25.6	55.9
Met	1.9	0.2	1.7	1.8	5.6	34.4
Ile	4.5	1.6	0.6	4.7	11.4	24.8
Leu	10.2	3.6	2.1	8.0	23.9	49.0
Tyr	2.9	1.5	1.4	10.3	16.0	26.3
Phe	4.1	1.7	1.0	10.8	17.6	23.4
His	3.0	3.8	3.1	7.4	17.3	27.5
Lys	9.6	12.4	2.8	11.6	36.4	60.2
Arg	12.4	4.6	1.2	4.5	22.7	55.8
Total	273.0	112.9	68.6	318.9	773.4	1736.6
HEATED DIET (B):						
Maillard products:						
Radioactivity (dpmx10^5 $(kg^{0.75})^{-1}$ day^{-1})						
	0	14.1	0.7	7.2	22.0	28.3
ε-DF-L (μmol $(kg^{0.75})^{-1}$ day^{-1})						
	0	243.0	8.7	0	251.7	309.8
Residual radioactivity (in lysine units, μmol $(kg^{0.75})^{-1}$ day^{-1})[2]						
	0	52.7	3.4	62.2	118.3	158.5
Amino acids[1] (μmol $(kg^{0.75})^{-1}$ day^{-1}):						
Asp	23.1	13.0	5.8	25.2	67.1	137.1
Thr	13.4	4.8	3.5	12.8	34.5	63.8
Ser	13.4	6.5	4.5	11.3	35.7	71.6
Glu	26.3	15.7	8.3	54.8	105.1	220.4
Pro	26.0	12.2	13.3	18.5	70.0	257.1
Gly	73.5	34.9	17.9	68.6	194.9	517.3
Ala	21.2	6.9	3.5	15.5	47.1	101.5
Val	8.1	13.7	4.2	4.8	30.8	71.6
Met	1.4	1.9	2.5	2.5	8.3	30.0
Ile	3.7	1.4	0.7	5.4	11.2	25.9
Leu	8.5	3.3	1.8	7.3	20.9	47.8
Tyr	2.4	1.9	0.9	8.8	14.0	30.4
Phe	3.5	1.1	0.8	7.7	13.1	25.9
His	2.6	4.5	4.9	5.5	17.5	34.0
Lys[3]	8.6	0	7.8	3.8	20.2	35.6
Arg	10.1	6.1	2.6	2.3	21.1	47.9
Total	245.8	127.9	83.0	254.8	711.5	1717.9

4.4 Discussion

4.4.1 The digestion of an early Maillard browned protein and absorption of amino acids and Maillard compounds

Digestion at the terminal ileum

Variation in the apparent digestibility of amino acids and nitrogen at the terminal ileum was generally very low for both diets, with an average coefficient of variation (CV) for amino acids (excluding glycine and lysine) of 1.1 %. In general the most variability was noted for the least digestible amino acids.

Casein in the non-heated diet was generally well digested at the terminal ileum and digestibility values agree well with other values reported for pigs (Zebrowska & Buraczewski, 1977; Low, 1979; and Kies *et al.*, 1986). The lower apparent digestibility for glycine, alanine, serine, threonine and aspartic acid, may be explained by the endogenous contribution, since secreted proteins are rich in these amino acids (Low, 1979).

Heat treatment, however, caused statistically significant decreases in the apparent ileal digestibility of nitrogen and several essential amino acids (lysine, threonine, valine, histidine and methionine) and non-essential amino acids (arginine, serine, glutamic acid and proline). Since the decreases were mostly small (less than 5 % units), only the decrease for acid hydrolysed lysine (39 % units lower), and possibly arginine (by 7 % units), may be of practical significance. A similar overall decrease in the apparent ileal digestibility of amino acids and nitrogen was reported (Plakas *et al.*, 1985) to occur in rainbow trout, fed fish protein isolate reacted with glucose under mild conditions (40 day incubation at 37 ^{o}C). Their lower lysine digestibility (by 19 % units) was also based on acid hydrolysis.

In the present study acid hydrolysed lysine overestimated the actual lysine (unblocked) in the heated diet, but did not in the non-heated diet. This leads to a problem in practical diet formulation for animals whenever processed proteins are used. A portion of the lysine appears to be present (by acid hydrolysis) but is nutritionally unavailable (Carpenter and

Booth, 1973). For these proteins total lysine by acid hydrolysis is usually considerably higher than FDNB reactive lysine (Erbersdobler and Anderson, 1983), as lysine is regenerated on acid hydrolysis of ε-DF-L. In these situations FDNB reactive lysine gives a better estimate of the nutritionally available lysine than the acid hydrolysed lysine estimates. However, in more severely heat damaged Maillard proteins, were there is actual destruction of lysine, there is a good agreement between FDNB reactive lysine and the total lysine obtained by acid hydrolysis (Erbersdobler and Anderson, 1983). The Maillard reaction in the present system resulted in some loss of lysine, that isn't totally accounted for by the regenerated lysine; this equaled 19 mmol/g Cr, i.e. 50 % of the actual lysine present in the non-heated diet. That is, some lysine was destroyed with the formation of advanced Maillard products. Lysine was also regenerated with acid hydrolysis of ileal digesta from the heated diet, and accounted for about 32 % of the acid hydrolysis lysine present. Although FDNB reactive lysine accurately determined the actual lysine present in both diets, this was not the case for the ileal digesta, particularly that for the heated diet. This is due to the form in which lysine is present, as the FDNB reactive lysine procedure only detects lysine present as C-terminally bound or internally bound. The other forms of reactive lysine, N-terminally bound or free, are not included in these estimates as they are extracted as di-dinitrophenol derivatives (Booth, 1971). Therefore, subtraction of FDNB reactive lysine from the actual lysine present indicates the amount of N-terminally bound or free lysine. In the present study this shows that the form of lysine and the amount present in ileal digesta from pigs fed the heated diet is considerably different to the non-heated diet, as the heated diet had 13 times more N-terminally bound or free lysine. Calculation of FDNB reactive lysine ileal digestibility coefficients would, therefore, be misleading as these would greatly overestimate lysine digestibility.

The lysine digestibility coefficients measured by acid hydrolysis appear to be similar to the actual lysine digestibility values for the heated diet (54 vs 59 % respectively). However, it must be recognized that actual lysine digestibility could range between 53 and 71 %; the two extremes of ε-DF-L behaviour (assuming it to be either protein bound or free). Since the acid hydrolysis estimates of lysine ileal digestibility for the heated diet fall within this range, is acid hydrolysis lysine adequate for prediction of actual lysine digestibility, without accounting for the regenerated lysine? In practice acid hydrolysis lysine digestibility is often

undertaken and accepted as an accurate measure. In an ideal situation, where actual lysine and ε-DF-L are absorbed to the same extent, and where the behaviour of ε-DF-L is similar in the diet and ileal digesta, acid hydrolysis lysine digestibility would be an accurate measure of the actual lysine digestibility. The situation in the present study, where ε-DF-L behaved as if protein bound, would also provide an accurate measure. However, from the present digesta chromatography results it would seem more likely that ε-DF-L behaved as present in the free form underestimation the actual lysine digestibility by 17 % units. Similarity between the above discussed estimates of lysine digestibility will also be greatly influenced by the dietary intake of ε-DF-L, in relation to the actual lysine intake. In the present study the ε-DF-L/AL ratio of the heated diet was 0.6. In practice where the ε-DF-L/AL ratio should be much lower than 0.6, it would appear that measurement of acid hydrolysis lysine digestibility may be a reasonably accurate indication of the actual lysine digestibility, particularly when the ratio of ε-DF-L /AL is low, i.e. < 0.25. It is further noteworthy that although the acid hydrolysed lysine ileal digestibility coefficients were much lower than the actual lysine ileal digestibility coefficients, the measures of lysine uptakes were very similar, i.e. 14.0 mmol/g Cr compared to 14.1 mmol/g Cr respectively. Therefore, acid hydrolysed lysine estimates can be used to accurately predict actual lysine availability, as the overestimate of dietary lysine and underestimate of lysine ileal digestibility from acid hydrolysed lysine estimates, fortuitously cancel in the present results.

In the present study the FDNB reactive lysine content of the heated diet (74 % of the total lysine by acid hydrolysis) overestimated the nutritionally available lysine determined by ileal digestibility (by 20 - 30 % units). Others reports (Erbersdobler & Anderson, 1983; and Plakas *et al.*, 1988) found than FDNB overestimated plasma lysine bioavailability (by between 20 and 30 %; and 14 %, respectively). However, it was recognized that this was a predictive measure of lysine bioavailability showing a good correlation with plasma responses. The overestimation of FDNB reactive lysine is because this method does not account for a decrease in digestibility of actual lysine. Therefore, in instances where there is a decrease in actual lysine digestibility and absorption (McBee & Marshall, 1978; Nordheim & Coon, 1984; and the present study), FDNB values will overestimate nutritionally available lysine.

As mentioned previously, the digestibility of actual lysine in the heated diet may range according to the two extremes of ε-DF-L behaviour. In either extreme, the apparent digestibility of actual lysine in the heated diet is well below (25 - 43 % units lower) the apparent digestibility of lysine in the non-heated diet. Thus, lysine would appear to be the first limiting amino acid in the heated diet. Plakas *et al.* (1985 & 1988) also showed lysine bioavailability to be the first limiting amino acid in an early Maillard browned protein, in trout, from the investigation of plasma lysine levels.

In the present investigation the values for the apparent ileal digestibility of Maillard products were generally more variable than those for amino acids and nitrogen, with ε-DF-L (CV = 21 %) being particularly variable. The digestion of Maillard products especially ε-DF-L, was much lower than for nitrogen and amino acids (except glycine and lysine). Total radioactivity was 70 % digestible at the terminal ileum, with ε-DF-L being less digestible (34 to 58 %) than residual radioactivity (73 to 82 %).

The low digestibility and absorption of ε-DF-L may be due to lack of a specific carrier. A recent report (Sherr *et al.*, 1989) examined the absorption of free ε-DF-L and ε,α-diDF-L using a portal vein catheterization technique in rats. It was determined that free ε-DF-L appeared to be absorbed by active transport, as possibly one of the free amino groups (α) was still recognized by the carrier. However, ε,α-diDF-L appeared to be absorbed by passive diffusion since both amino groups were blocked. As ε-DF-L in the present study was likely to be bound in small peptides, possibly having the α-amino group also blocked (refer chromatography results), it seems likely that it will be absorbed by passive diffusion. This would partially explain the low apparent digestibilities noted. Since the observation that ε-DF-L has some affinity for gut wall components (Erbersdobler, 1977 and Sherr *et al.*, 1989) cannot be ruled out, the apparent ileal digestibility of ε-DF-L may have been overestimated, as although taken up by the mucosa the majority may re-enter the small intestine lumen at a later time compared to the sampling time used. This possibility may also hold for premelanoidins and other Maillard products.

It was assumed (see Chapter 3) that the residual radioactivity was due to the presence of advanced Maillard products. Finot and Magnenat (1981) have indicated a lower digestibility

of advanced Maillard products compared to ε-DF-L. The present results do not agree with this. However, Finot and Magnenat (1981) used enzymatically digested and semi-purified premelanoidins from a casein-glucose mixture, so their results may not be directly applicable to the present work. Alternatively, the present dietary residual radioactivity values may indeed be overestimates of the radioactivity attributable to advanced Maillard products, because of ^{14}C-glucose and/or its decomposition products (such as caramel) adsorbing onto protein and, furthermore, some ^{14}C-glucose may be also weakly associated with certain amino acids (other than lysine) in the intact protein, but recovered after acid hydrolysis (refer discussion, Chapter 3). The effect of acid in the stomach and proteolytic hydrolysis may have been sufficient to release a proportion of these weakly associated radioactive compounds. These compounds being sugar based would be readily absorbed, resulting in an overestimate of the digestion of advanced Maillard products.

Molecular weight distribution of compounds (peptides, amino acids and Maillard products) in ileal digesta and its significance

As discussed, the heat treatment of the casein-glucose mixture caused significant decreases in the ileal digestibility of some essential amino acids (particularly lysine) and non-essential amino acids. Furthermore, the digestibility of Maillard products, particularly ε-DF-L was low. Limit peptides containing Maillard compounds may account for these decreases.

Gel chromatography of the acetic acid soluble ileal digesta resulted in reasonably high recoveries (80 %) for all amino acids. The unrecovered 20 % would be mainly associated with the insoluble fraction (possibly in mucoproteins). The higher recoveries of Maillard products indicated that these are associated in the soluble fraction.

In the present investigation, the ileal digesta of pigs fed the non-heated diet contained approximately twice as many amino acids in peptides (67 %) than as free molecules. Individual amino acids were distributed in peptides corresponding to 2 - 3, 7 and above 10 residues in length, in approximately the same proportions. Abidi and Mercer (1973) also reported a higher proportion of amino acids in peptide form (77 %) than as free molecules in the water soluble fractions of ileal digesta for humans fed high protein diets. Their observations indicated that most peptides were only 2 - 3 residues in length.

Amino acids in the terminal ileum digesta are from both exogenous and endogenous origins. Although the distinction was not made in the present investigation, a previous study (Kies *et al.*, 1986) on the digestion of casein in pigs (which accounted for the endogenous amino acid pool in ileal digesta by subtracting amino acids found after feeding a protein free diet), showed that the majority of dietary amino acids (lysine, histidine, arginine, aspartic acid, proline, glutamic acid, alanine, methionine, leucine and tyrosine) were completely digested by the terminal ileum. They concluded that intact lactic casein is a good control protein source for these purposes in nutritional experiments in growing pigs. In light of their results, amino acids in the ileal digesta from the non-heated diet can be considered as being mainly of endogenous origin, thus providing a good baseline for the effect of heat treatment on the amino acid composition of ileal digesta from the heated diet.

The molecular weight distribution in the ileal digesta from pigs fed the heated diet indicated that the increased amino acid content of the ileal digesta could be totally accounted for by the increased peptide content, particularly of 2 - 3 residues in length, of about 430 MW. In this region, the ratios of ε-DF-L and actual lysine to the combined total of amino acids was 1 : 2.5 and 1 : 3.1 respectively. This suggests that ε-DF-L and unblocked lysine may be components of the high proportion of the di and tripeptides present, and that they both may be present in the same peptides. On a molecular weight basis this is a reasonable hypothesis as the average molecular mass of amino acids is about 130 and for ε-DF-L is 308; a molecule of water would be eliminated with each peptide bond present. Furthermore, the proportion of Maillard products (ε-DF-L and residual radioactivity) to "extra" amino acids (by subtracting those present in the ileal digesta from the non-heated diet) equates to approximately 1 : 1. The sequences and relative proportions of the four chains which constitute casein (α_{s1}, α_{s2}, β and κ) in bovine milk are known (Eigel *et al.*, 1984). Since casein contains several sequences with two lysine residues either adjacent to each other or only separated by one other amino acid, di and tripeptides containing both ε-DF-L and an unblocked lysine residue are highly possible products from the digestion of the early Maillard browned casein used in this study. It is also probable that certain lysine containing sequences may be particularly susceptible to the occurrence of the Maillard reaction. This may explain the relatively high content of valine, arginine, proline and methionine in these

peptides compared to the amounts present in the corresponding fractions from the ileal digesta of animals fed the non-heated diet. The four amino acids listed above also had reduced ileal digestibility coefficients in the animals fed the heated diet.

Peptides containing Maillard products could also be resistant to enzymatic hydrolysis, possibly because of the altered side chain when ε-DF-L, or other Maillard products, are attached to a lysyl group, or because the bulky nature of these constituents could cause steric hindrance. A further possibility is that cross-linkages could have occurred between the Maillard products and side chains of adjacent amino acid residues either in the same peptide or in different peptides. Some types of advanced Maillard products are particularly likely to be highly reactive in these cross-linkages, such as pyrazine type radicals and their interactions (Namiki *et al.*, 1977). Such cross-linkages and other enzymatically resistant bonds have been also suggested to occur in browned proteins, by many other workers (Buraczewski *et al.*, 1967; Bocter & Harper, 1968; Nesheim & Carpenter, 1967; Valle-Riestra & Barnes, 1970; Ford & Shorrock, 1971; and Mori & Nakatsuji, 1977), and to be responsible for the reduction in digestibility.

It is also possible that the high content of ε-DF-L and other Maillard products in these peptides, and possible cross-linkages, may sterically hinder the reaction of FDNB with ε-NH_2 groups. Thus, the low FDNB reactive lysine content of the ileal digesta from pigs fed the heated diet, may be due partly to some internally or C-terminally bound lysine having been prevented sterically from reacting with FDNB.

The production of peptides with enzymatically resistant peptide bonds may also reduce amino acid and peptide absorption. Digestion of protein in the intestinal lumen and brush border mucosa produces a mixture of small di and tripeptides and amino acids of which the former predominate (Matthews, 1975). This has been shown for a variety of species including man (Abidi and Mercer, 1973). Matthews (1975) has reviewed the intestinal absorption of peptides. In summary, peptide and amino acid absorption occur under independent systems. The uptake of amino acids in peptides is greater than for free molecules and involves either of two mechanisms: (i) - Intracellular hydrolysis, by the transport of peptides into epithelial cells were they are hydrolysed, and/or (ii) - Intramembrane hydrolysis, where the hydrolysis

of peptides occurs in the membrane of epithelial cells, with the resultant amino acids being transported in the usual manner. The structural requirements for peptide absorption are poorly understood, however, competitive studies have demonstrated both specific and non specific mechanisms, with the former being the most important.

The presence of ε-DF-L and other Maillard compounds in small di and tripeptides, as well as free molecules, may also affect the absorption and hydrolysis of other peptides by competing for the same transport mechanisms (Erbersdobler, 1977; Lee *et al.*, 1977 A; and Sherr *et al.*. 1989). A recent study (Sherr *et al.*, 1989) has shown that the presence of free ε-DF-L competed with lysine for the absorption carrier, whereas free ε,α-diDF-L appeared to block the absorption site rather than compete for the carriers. An earlier study (Erbersdobler, 1977) provided evidence for absorption by passive diffusion of ε-DF-L and showed that ε-DF-L had a considerable affinity for gut wall components, with its presence leading to a decrease in the transport of neutral short chain amino acids (threonine, proline and glycine) but to have no affect on the uptake of lysine, methionine and galactose; the effect on uptake of peptides was not reported

A further explanation for the observed decrease in apparent ileal digestibility may be an increased endogenous protein loss. Since, the heated protein was shown to be less digestible, the gastrointestinal tract may have adapted to the indigestible source by an increased secretion of digestive enzymes (Buraczewski *et al.*, 1967; Percival & Schneeman, 1979; and Schneeman & Dunaif, 1984). However, the amino acid endogenous contribution of digestive enzymes is relatively small and seems more likely to be due to an increases in mucus and cell loss. Consequently, the resultant degradation products of endogenous secretions may contribute to the residual peptide pool in digesta at the terminal ileum.

In summary, results from the present study indicate that the decrease in apparent ileal digestibility of the early Maillard browned casein can be probably explained by the presence of enzymatically resistant peptide bonds due to the formation of Maillard products, and limit peptides accumulated in the small intestine. These limit peptides, and also free Maillard products such as ε-DF-L, may also adversely affect amino acid and peptide transport mechanisms. In addition there may be an increase in endogenous amino acid loss.

Digestion over the entire digestive tract and the influence of the large intestine

Variations in estimates of the apparent digestibility of amino acids, nitrogen and Maillard products over the entire digestive tract were generally low and similar to variations reported for ileal digestibility (CV = 1.1 %).

It should be noted that the ileal/faecal comparison for the digestibility of the heated diet is between two somewhat different early Maillard browned diets (refer Chapter 3). The heated diet (B) was used over the faecal collection period, whereas, the ileal digestibility estimates were from ingestion of the radioactively labelled heated diet (BR). Although results in Chapter 3 show that the two casein-glucose mixtures used in these diets were very similar in their extents of the Maillard reaction, differences may exist. In addition the heated mixture was supplemented to replace the lysine and glucose lost, whereas the radioactively labelled mixture was not, and this also may have influenced the present results.

In the present investigation the action of microorganisms in the large intestine caused a significant disappearance of most amino acids and nitrogen for both diets, and Maillard products (especially ε-DF-L) for the heated diet The average differences in the calculated apparent ileal and faecal digestibilities of amino acids in the present study for the non-heated diet are similar to those from other studies for casein diets (Table 4.16). The 8 % increase in nitrogen digestibility in the large intestine in the present study was also the same as that reported by Zebrowska and Buraczewski (1977).

Table 4.16 *The average apparent ileal and faecal digestibility coefficients (%) of amino acids in casein from the non-heated diet,the differences between them and comparison with casein diets from other studies*

Digestibility (%)			
Ileal	Faecal	Difference	Reference
91.2	97.2	6.0	Present study
89.4	93.8	4.4	Ivan & Farrell (1976)
88.7	94.4	5.7	Zebrowska & Buraczewski (1977)
94.0	96.8	2.8	Low (1979)

The largest differences between ileal and faecal estimates (for both the non-heated and heated diets) were noted for the components with the lowest ileal digestibilities (correlation = 0.99). Consequently, the significant differences in digestibility of amino acids between the non-heated and heated diets found at the terminal ileum were not detected in the faeces. Amino acids in the heated diet (excluding glycine and lysine) were further digested (6.1 % units) in the large intestine, whereas the same amino acids from the non-heated diet were not digested to the same extent in the large bowel (only a further 3.9 % units). These differences were statistically significant except for methionine, isoleucine and tyrosine. Net synthesis of methionine, arginine, cystine and tyrosine (Austic, 1983) in the large intestine affects their digestibility values. This lack of sensitivity of faecal digestibility estimates to differences in digestibility, through the influence of the large intestine, has been stressed in a recent review on the digestion of amino acids in pigs (Sauer & Ozimek, 1986). They concluded that there is a modifying and apparent equalizing effect due to the microorganisms of the large intestine. The present results support this by demonstrating significant differences between the ileal and faecal digestibilities, to the extent where differences between diets were not detectable using faecal estimates.

Many other researchers have demonstrated increases in the faecal excretion of amino acids in rats fed Maillard browned proteins (Henry and Kon, 1950; Boctor and Harper, 1968; Sgarberi *et al.*, 1973; Tanaka *et al.*, 1977; and Knipfel, 1981). However, these studies used more severely heat damaged proteins which presumably contained a higher proportion of cross-linkages and advanced and final Maillard products than were present in the early Maillard browned casein in the present study..

The large intestine played a major role in the apparent digestibility of Maillard products, especially ε-DF-L, which was digested more than was the residual radioactivity. This was also demonstrated by the changes in the proportions of Maillard products from the terminal ileum to the faeces, with ε-DF-L accounting for 45 and 14 % of the total remaining radioactivity respectively.

The ε-DF-L entering the large intestine can be utilized by the micro-organisms to supply lysine, as they possess the appropriate mechanisms to hydrolyze the attached sugar moiety

(Tanaka *et al.*, 1975 B; Horn *et al.*, 1968; and Finot, 1973). The residual radioactivity fraction, considered to be mainly advanced Maillard products, is unlikely to be utilized as well as the ε-DF-L by the microflora, as these Maillard products have undergone extensive chemical reactions where rearrangement of the lysine residue has occurred. However, advanced Maillard products are metabolized by the microflora with the production of CO_2 (Finot & Magnenat, 1981)

The low ε-DF-L faecal excretion of 3.4 % units of that ingested agrees well with the value found by Finot and Magnenat (1981) for fructosyl-lysines in milk proteins, when these were fed to rats. However, the excretion of advanced Maillard products is dependant on the proportions of premelanoidins and melanoidins. Advanced Maillard products from ^3H labelled lysine in casein were 47 % excreted in the faeces (Finot and Magnenat, 1981). Their further studies using premelanoidins and melanoidins resulting from the reaction between casein and glucose showed 64 % and 87 % excretion respectively. These latter studies used enzymatically hydrolysed and fractionated compounds, which were much more severely heat damaged and of a higher purity than the samples used in the present study. The heated casein-glucose mixture used in the present study, although brown, was considered to be an early Maillard browned protein (refer Chapter 3), with advanced Maillard products being mainly premelanoidins. However, the actual concentrations were not determined. In the present study 7 % of the ingested residual radioactivity was excreted in the faeces.

The patterns of excretion of radioactivity in the faeces, found in the present study, were similar to those documented on feeding an early Maillard ^3H-lysine labelled casein to rats (Finot & Magnenat, 1981), but levels of excretion in the latter were higher (13.5 vs 6.3 %), which is probably due to differences in labelling techniques. Since the microorganisms in the large intestine can utilize early Maillard products efficiently, these processes would have incorporated the ^3H into the microorganisms which were then excreted.

Digestion in sections of the small intestine

Retention times of digesta and the digestibility values for samples collected in different segments of the small intestine were determined on the analysis of pooled samples, the

differences should be considered in light of the dry matter recoveries and their variability, and be interpreted with caution.

The distribution of dry matter over sections of the small intestine were highly variable (CV = 20 %). However, the total recoveries were less variable in the stomach and over the entire small intestine (CV = 15 %). The dry matter distribution and calculated retention times indicated that the heated diet had an apparently faster rate of stomach emptying, but elicited a slower small intestinal motility compared to the non-heated diet. However, overall a similar transit time from the stomach to the terminal ileum was found for both diets.

In contrast, a decrease in the rate of stomach emptying has been observed by Tanaka *et al.* (1977) and Kimiagar *et al.* (1980) on feeding browned egg albumin to rats. Gastric emptying is a discontinuous process which is affected by the amount ingested and the composition of the diet, particularly the nature and amount of ingested protein (Rerat, 1981). The denaturation of milk proteins due to heat treatment reduces the ability of bovine milk proteins to form a clot in the stomach (Braude *et al.*, 1976), which in turn causes an increase in the rate of stomach emptying (Kaufman, 1984). The present results appear to be consistent with this phenomena.

The apparently slower transit times and significantly greater recovery of dry matter over the small intestine for the heated diet, indicates that because the heated diet is less digestible it remains in the small intestine for a longer period. In contrast, Leibholz (1984) using pure proteins, with different digestibilities, showed that the source of protein did not influence the retention time of digesta in the small intestine. However, none of the proteins examined were Maillard heat damaged proteins.

Virtually complete digestion and absorption of dry matter, amino acids (except for glycine and lysine) and nitrogen was shown for both the non-heated and heated diets; approximately 30 - 50 % of the way along the small intestine proximal to the pylorus. These results agree with Leibholz (1984) who found that the major site of nitrogen digestion and absorption for a milk diet was between 25 and 50 % proximal to the pylorus. Similarly, Low (1979) found that digestion and absorption of amino acids and nitrogen from casein and potato flakes was

virtually completed between 43 and 76 % of the way along the small intestine. From the results of the present study there were no apparent differences between the non-heated and heated diets in the pattern of digestion of dry matter, amino acids (except lysine) and nitrogen. Lysine digestibility showed a major reduction due to heat treatment. As discussed previously, the actual lysine values are greatly influenced by the ε-DF-L content whether it is free or bound. However, the reason for the negative digestive pattern for actual lysine along the gastrointestinal tract is uncertain. The digestion of Maillard products, particularly ε-DF-L, also showed a negative trend.

A comparison of the amounts of Maillard products in the sixth small intestinal section and in digesta collected at the terminal ileum was important in that it showed the possible effect of a sloughing epithelium. Samples from the small intestinal sections were taken 30 min after slaughter, whereas, digesta samples at the terminal ileum were taken immediately. The amount of amino acids, nitrogen, dry matter and Maillard products in the contents of these two samples should be the same as digestion is the same over terminal portions of the small intestine (Low, 1979; Leibholz, 1985; & Kies *et al.*, 1986). Since the sixth intestinal section contained much higher amounts of compounds, particularly ε-DF-L, than that present in terminal ileal digesta, the results indicate an accumulation of ε-DF-L compared with amino acids and nitrogen in the mucosal walls. Furthermore, from the distribution of Maillard products in the contents and mucosa walls there is a greater amount in the lower sections of the small intestine than the upper sections, with higher percentages in the respective mucosa of these lower regions. Thus, contribution of Maillard products from the sloughed mucosa may offer an explanation for the observed negative digestive patterns of ε-DF-L. Assuming that the contribution from the sloughed mucosa is of the same magnitude in the various sections of the small intestine, then because the concentrations in the mucosa are higher in the more proximal sections of the small intestine, the digestibilities will become less and, therefore, appear to have a negative trend along the gastrointestinal tract; since the chromium is not retarded and passes at a normal rate. This possible accumulation and retardation in the mucosa wall of Maillard products, especially ε-DF-L would explain the present results. In support of this, fructosyl-lysine has been documented to have a considerable affinity for gut wall components and was shown to be absorbed by passive diffusion (Erbersdobler, 1977 and Sherr *et al.*, 1989).

4.4.2 The metabolic transit of amino acids and Maillard compounds following the ingestion of an early Maillard browned casein

The analysis of portal and jugular serum positively identified, not only ε-DF-L, but also other ^{14}C-radioactive compounds which accounted for a major portion (60 %) of the total radioactivity in the serum. Plakas *et al.* (1985) also reported ε-DF-L in the plasma of rainbow trout given Maillard browned proteins. It is not known from the present results whether ε-DF-L in serum was present either as a free molecule or with a peptide fragment. Although the results indicate that Maillard compounds were absorbed, without measurements of blood flow, total volumes and several sampling intervals, the concentrations would not be directly related to dietary intake levels. Sherr *et al.* (1989) also detected ε-DF-L and ε,α-diDF-L in portal blood from a duodenal infusion of these free molecules.

Consumption of the heated diet did not cause a significant change in the excretion of amino acids (except for increased lysine), compared to the non-heated diet, but the data were highly variable. The present results infer that if amino acids other than lysine are absorbed in a non-utilizable form they are of minor nutritional significance as they increased by less than 3 % units of than absorbed, contributing a negligible portion to the reduction in nutritive value of early Maillard browned casein.

The pattern of radioactivity excretion in the urine was similar to that reported when an early Maillard ^{3}H-lysine labelled casein was fed to rats (Finot &Magnenat, 1981), but the amount excreted was less (10 % vs 20 % excretion); once again this may be a product of the different labelling techniques. The present results, however, agree with those obtained from feeding rats manufactured milk powders (Finot & Magnenat, 1981), spray-dried casein-glucose mixtures (Finot & Magnenat, 1981) and a U^{14}C-lysine labelled casein-glucose browned system (Mori *et al.*, 1980). About 20 % of the ingested residual radioactivity was estimated to be excreted in the urine. This may reflect the excretion of premelanoidins and melanoidins present in the heated casein-glucose mixture (heated diet) as Finot and Magnenat (1981) have shown that ingested semi-purified premelanoidins and melanoidins were present in the urine (23 - 30 % and 2 - 7 % respectively). However, parts of the residual

radioactivity excreted, in this study, may be also due to the presence of radioactive non-Maillard compounds mentioned previously, such as decomposition products of ^{14}C-glucose. This may also be a factor in explaining the change in the proportion of total radioactivity contributed by ε-DF-L in the urine (28 %) and in the serum (35 - 42 %), as such decomposition products probably would be water soluble and excreted readily. A further possibility is that some residual radioactivity may have been absorbed from the large intestine. Finot and Magnenat (1981) have shown that the microflora of the large intestine can efficiently utilize ^{14}C-ε-DF-L and ^{14}C-premelanoidins to a lesser extent, and that this results in the expiration of $^{14}CO_2$ (35 % and 7 % respectively). Some of the $^{14}CO_2$ absorbed from the large intestine could also be lost in the urine as ^{14}C-urea and so contribute to the residual radioactivity excreted.

Estimation of the urinary excretion of the absorbed Maillard products indicate that 83 % of the total radioactivity, 64 - 83 % of the ε-DF-L and 71 - 78 % of the residual radioactivity remain unaccounted for. However, since the digestibilities of these Maillard products were possibly overestimated (as discussed previously), the amounts unaccounted for during metabolic transit may also be overestimated. Several reports (Erbersdobler, 1977; Finot & Magnenat, 1981; and Sherr *et al.*, 1989) argue that ε-DF-L and other Maillard products are not metabolized in tissues. Even so, some storage of these compounds may have occurred as radioactivity has been detected in body tissues from various radioactively labelled Maillard proteins (Finot & Magnenat, 1981). Furthermore, both ε-DF-L and ε,α-diDF-L have been shown (Sherr *et al.*, 1989) to be present in liver microsomes, but the significance of this is uncertain.

The molecular weight distribution of peptides, amino acids and Maillard products in urine

The recovery of urinary amino acids after chromatography was very low for both diets (40 to 50 %) compared with the recovery of Maillard compounds (80 %). This may have been a result of the freeze-drying causing an irreversible association which would not dissociate in 0.2 M acetic acid. A particular hydrophobic fraction of the urine, containing a portion of the total amino acids, may have also adsorbed onto the Sephadex gel matrix. The urine samples were applied to a freshly regenerated column which became discoloured from their application, both from the non-heated and heated diets. In view of these low (but

comparable) recoveries for both diets, it must be stressed that the amino acid distribution is incomplete and does not account for the total amino acid composition of urine.

The elution patterns of the urine samples from the two diets appeared to show obvious differences in their respective compositions, but the amino acid distributions were similar. Nevertheless, heat treatment to casein caused an increase in the excretion of two groups of peptides corresponding to either 2 - 3 residues in length (about 430 MW) or 4 - 5 residues in length (about 715 MW), with the latter containing the majority of Maillard products, especially ε-DF-L. This appears to be in contrast to the suggestion that the ε-DF-L units in urine are in the free form and not peptide bound (Ford & Shorrock, 1971; Mori *et al.*, 1980; and Finot & Magnenat, 1981). Furthermore, the proportion of Maillard compounds to "total" amino acids in the 715 MW peptides fraction (2 : 1) infers that some peptides are mainly composed of Maillard products. However, the proportion of Maillard compounds to "extra" amino acids (by subtracting those in the same region from the non-heated diet) equates to 20 : 1. These also contained relatively large amounts (in comparison with other asmino acids) of proline, valine and methionine; the amino acids which were also more abundant in the Maillard product containing ileal peptides. It is of interest that the apparent size of these peptides is larger than those containing the majority of Maillard products present in ileal digesta (molecular weights of about 715 and 430 respectively). However, in the ileal digesta, the adjacent region of larger peptides also contained Maillard products.

Regenerated lysine was overestimated in the urine from the heated diet, as there was more calculated than the acid hydrolysed lysine present in the 715 MW peptide region. As shown in Chapter 3, the behaviour of ε-DF-L was dependant on the composition of the hydrolysis medium. In different physiological samples the medium changes considerably, particularly for urine, containing many complexed organic molecules with properties which may cause a change in the proportions of acid hydrolysed products released from the hydrolysis of ε-DF-L. The 0.1 % phenol included in the acid hydrolysis mixture, caused an increase in the lysine regenerated and a decrease in the furosine formed (refer Chapter 3). If the action of phenol was impaired by the compounds present in urine, the regenerated lysine would be overestimated. This may explain the present discrepancies. However, the volume of acid to sample ratio should have been sufficient to eliminate this effect. It could also be that the

assumption of ε-DF-L behaving as a free molecule maybe inaccurate, overestimating regenerated lysine.

As 10 % of the total radioactivity in urine appeared to be associated with urea, this suggests that either some of the Maillard products can be metabolized, or that some of the residual radioactivity were from other compounds which could be incorporated into urea. For example these could be non-Maillard product sugar fragments discussed previously, or they could be radioactivity compounds absorbed in the large intestine. Mori *et al.*, (1980) demonstrated that the radioactivity in the urine of rats fed browned U^{14}C-L-Lysine labelled casein could be separated into three components, 50 - 60 % as ε-DF-L, 20 - 25 % as other ninhydrin positive compounds and the remaining fraction as ninhydrin negative compounds. These may be contributed from other absorped Maillard products such as premelanoidins, metabolized components of lysine, or possibly metabolized Maillard products.

The colour of the urine between the treatments was quite different. On chromatography the reddy-brown pigments appeared to be associated with Maillard products, corresponding to the 715 molecular weight Maillard peptide fraction. Some additional information on these pigments was obtained in an independent study from a second purification using HPLC chromatography. The pigments eluted in the hydrophobic regions of the HPLC gradient and were not associated with radioactivity. Thus, these pigments are unlikely to be due to Maillard products. There have been no other reports of discoloured urine from Maillard browned pigments from a dietary origin. This is also supported by the studies of Homma and Fujimaki (1981) on the growth responses of rats fed a diet containing non-dialyzable melanoidins. In their studies they showed from colorimetric measurements that melanoidins were excreted in negligible proportions in the urine. But from that present, their data indicated that dietary melanoidins were degraded into smaller non-coloured materials through digestion and absorption in the intestines of rats. Analysis of these urinary brown pigments and their significance are discussed in greater detail in Chapter 5.

4.4.5 Summary

From the present study with young growing pigs it is concluded that:

(1) Heat treatment caused a significant decrease in the digestibility of several non-essential and essential amino acids at the terminal ileum, which could be accounted for by the formation of small limit peptides of 2 - 3 residues in length (430 MW). These appear to be associated with Maillard compounds, particularly ε-DF-L.

(2) The large intestine further digested residue form the terminal ileum to the extent where significant differences, due to heat treatment, in the faeces were not detectable. This stresses the equalizing effect of the large intestine, particularly on the less digestible material at the terminal ileum and indicates that it is inadequate for assessing the digestibility and availability of amino acids, nitrogen and Maillard compounds.

(3) There was no major change in the urinary excretion of free amino acids, which indicates that the nutritive value of the heated diet was not impaired due to the absorption of unavailable amino acids which were directly excreted.

(4) The composition of limit peptides indicated that the most limiting amino acids were actual lysine, valine, methionine and proline. They also appeared to be associated with Maillard products particularly in ileal digesta, and to a small extent in the urine.

(5) A summary of the fate of Maillard products expressed as a percentage of that ingested per day per metabolic body weight is shown in Table 4.17.

Table 4.17 Estimates for ε-DF-L and residual radioactivity may range between two values depending on whether ε-DF-L is present as either a (**F**) free molecule or (**P**) protein bound . In situations where it was uncertain they were calculated as a (**C**) combination. It seems likely that the ε-DF-L in ileal digesta will behave as if present as a free molecule from the Sephadex G-15 chromatography results (refer, 4.3.4).

Table 4.17 *Dietary intake levels of Maillard compounds (total radioactivity, ε-DF-L and residual radioactivity) and tabulation of the fate of the Maillard compounds expressed as a percentage of that ingested.*

	Total radioactivity	ε-DF-L	Residual radioactivity
Dietary intake (units $(kg^{0.75})^{-1}$ day			
	3.30×10^7 dpm	2.46 mmol[P]	2.48×10^7 dpm[P]
Fate of Maillard compounds as a percentage of that ingested:			
Absorbed from small intestine	70	49[C] (34[F] - 58[P])	77[C] (73[P] - 82[F])
Excretion in urine	10	11[F]	20[F]
Loss through metabolic transit	60	38[C] (23[F] - 47[P])	57[C] (53[P] - 62[F])
Amount present in leal digesta	30	51[C] (42[P] - 66[F])	23[C] (18[F] - 27[P])
Disappearance in large intestine	24	48[C] (39[P] - 63[F])	16[C] (11[F] - 20[P])
Excretion in faeces	6	3[F]	7[F]

Chapter 5

PHYSIOLOGICAL RESPONSES TO THE SHORT-TERM INGESTION OF AN EARLY MAILLARD BROWNED PROTEIN BY THE GROWING PIG

In the previous Chapter an early Maillard browned casein was found to have an impaired *in-vivo* digestibility. Further, Maillard products were absorbed and excreted in the urine, but with a proportion of the absorbed compounds remaining unaccounted for. The following Chapter concerns physiological responses of the pig to the ingestion of the Maillard compounds from an the early Maillard browned casein.

5.1 Introduction

As summarized in Chapter 1, the reduced nutritive value of protein heated in the presence of reducing sugars cannot be completely accounted for by the loss or unavailability of amino acids. In addition, Maillard compounds may have inhibitory, antinutritive or toxic properties (Adrian, 1974; Kimiagar *et al.*, 1980; Lee & Chichester, 1983; Pintauro *et al.*, 1983; and O'Brien & Walker).

Lee and Chichester (1983) argued that any detrimental effects of Maillard browned compounds on an animal, which cannot be directly related to a nutritional response, may be considered as being a "potentially" toxic response. In practice it is difficult to separate nutritionally related effects from toxic effects, as both can elicit similar physiological responses. A further complicating factor (Kimiagar *et al.*, 1980) is that the presence and action of inhibitory or anti-nutritive compounds cannot be readily detected using short term nutritional assays.

Adrian (1974) reported that the soluble fraction of a glucose-lysine reaction mixture (premelanoidins) reduced the protein efficiency ratio of casein when fed to rats for four

weeks. These premelanoidins were found to decrease the PER by reducing dietary nitrogen digestibility and the nutritional availability of nitrogen from casein. Similarly, Lee *et al.* (1976) found decreased growth rate and food conversion efficiency in rats after 4 weeks of feeding browned apricots added to a casein diet. Gastro-intestinal adaptive responses have been reported after just 10 days when feeding severely heat damaged pure casein to rats (Percival & Schneeman, 1979). Increased activities of trypsin and chymotrypsin and an increase in the protein content of the small intestinal digesta were found. Schneeman and Dunaif (1984) reported similar findings when feeding Maillard browned dried milk to rats. They suggested that because the rats were being fed an inadequate dietary protein, in the context of the limited availability of amino-acids due to heat treatment, there was a need for adaptation of the digestive system to the indigestible material. This may also be the cause for the decrease in protein efficiency ratios reported by Adrian (1974) and Lee *et al.* (1976). Consequently, it appears that the poor nutritional quality of heat-treated proteins may also be due, indirectly, to adaptive physiological responses of the gastrointestinal tract. Adrian (1974) further reported that premelanoidins reduced the digestion of casein *in-vitro*, suggesting an inhibition of the digestive enzymes. Extensive studies with the rat, using the low molecular weight fraction of a glucose-lysine reaction mixture not only showed interference with the intestinal digestion of egg white protein *in-vivo* (Oste & Sjodin, 1984), but also inhibition of carboxypeptidase A and aminopeptidase N *in-vitro* (Oste *et al.*, 1986 & 1987). Previous reports have shown that Maillard browned proteins may also delay the rate of gastric emptying (Kimiagar *et al.*, 1980), and it has been suggested (Schneeman & Dunaif, 1984) that holding a large volume of food for a prolonged period in the stomach may cause hypertrophy of the organ. This may subsequently change the gastric proteolytic enzyme activities.

Liver and kidney function are important in the detoxification, metabolism and excretion of absorbed non-utilizable material. A common screening procedure for possible damage to the liver is the determination of serum levels of various enzymes (Moss *et al.*, 1986): (i) Endogenous enzymes released after damage to hepatocytes (alanine transaminase, ALT and aspartate transaminase, AST); and (ii) Endogenous enzymes synthesized at an increased rate (alkaline phosphatase, AP and γ-glutamyl transpeptidase γ-GT). The enzyme ALT is more specific to liver damage than AST. Levels of AST also increase in response to myocardial infarction. The enzyme γ-GT is the most sensitive of the tests for liver damage and when used in conjunction with the determination of the other

transaminase activities can distinguish whether the damage is localized to the liver or heart. A further useful diagnostic test of liver and kidney function is the blood urea nitrogen and creatinine concentrations and their ratio. Blood glucose concentrations in serum can also indicate physiological stress from nutritionally inadequate diets (Root & Bailey, 1968). Rats fed a browned egg albumin for 3 months (Kimiagar *et al.*, 1980), showed increases in their relative liver and kidney weights and serum levels of the transaminases, alkaline phosphatase, urea nitrogen and glucose were elevated. Furthermore, the kidneys of rats (Schneeman & Dunaif, 1984) were heavier after a heat-damaged dried milk was fed to the animals for 4 weeks.

The overall objective of the present study was to determine if physiological responses in pigs consequent upon their ingestion of relatively large amounts of an early Maillard browned casein over a short (26 day) time period. The specific aims were:

(1) To assess if there is an effect of dietary Maillard compounds on protein digestive physiology in pigs given an otherwise nutritionally balanced diet.

(2) To determine if absorbed Maillard compounds impaired liver or kidney function in the young growing pig.

5.2 Experimental

The present study was part of that described in detail in Chapter 4 (Diet formulation 4.2.1; Animals and housing 4.2.2; General conduct of trial 4.2.3; and Sample processing 4.2.4). Additional aspects of the experimentation are described in the following sections.

5.2.1 Sampling and sample processing

The liver, right-hand-side kidney, pancreas and large intestine were removed from the euthanased animal and placed on a tray of ice. The large intestine was opened and residual material removed. The organ was washed with isotonic saline, blotted-dry and weighed. Extraneous tissue was removed from the liver (the gall-bladder emptied of its contents), kidney and pancreas, the tissues blotted dry and weighed and the pancreas

Table 5.1 [1]Analytical techniques are described in detail in Chapter 2: enzymatic analysis of pepsin, chymotrypsin, trypsin, alanine transaminase, aspartate transaminase and γ-glutamyl transpeptidase (2.2.7); protein by the modified Lowry method (2.2.9); creatinine based on the Jaffe reaction and urea based on the Fearon reaction (2.2.9); glucose by the direct Hexokinase method (2.2.2); and routine urinalysis tests; dipstick tests for bilirubin and urobilinogen, osmolality, specific gravity, Erhlic's test for hexosamines, fluoresence test for porphorins, indicans test, alkaptoneuria test (homogentisic acid), iron content by atomic absorption and spectrophotometry between 340 and 600 nm (2.2.9).

immediately frozen. The pH and weight of the contents from the stomach and caecum were recorded.

The pancreatic tissue was further processed. The tissue was diced while still frozen, weighed and homogenized (4 x 1 min pass) in ten times its weight of isotonic saline (Nitsan & Leiner, 1976). The homogenate was centrifuged and the supernatant frozen. A portion of the homogenate from the mucosa of the third intestinal section was centrifuged at 27,000 g for 10 min (0 - 10 °C), the supernatants filtered through glass wool and stored separately.

5.2.2 Laboratory analysis

A summary of the analyses conducted on samples is given in Table 5.1. The standard analytical techniques are described in detail in Chapter 2.

Table 5.1 *A summary of the chemical analyses used on tissue and body-fluid samples taken from the pig.*

Sample		Assay[1]
Stomach:	contents	Pepsin.
	mucosa	Pepsin.
Small intestinal mucosa (J3):		Aminopeptidase N.
Ileal digesta:		Chymotrypsin, trypsin, protein.
Pancreas:		Chymotrypsin, trypsin, protein.
Urine:		Creatinine, urea, routine urine analysis tests.
Serum:	jugular	Creatinine, urea, glucose, aspartate transaminase, alanine transaminase, γ-glutamyl transpeptidase.

5.2.3 Statistical analysis

All statistical analyses were carried out using a one-way analysis of variance. Liveweight gain over the period of the trial was fitted as a covariate.

5.3 Results

Over the 26 day period of the trial all pigs remained healthy except for pig #1, fed diet B. The latter pig had a reduced appetite and elevated temperature. Samples were taken for this animal but the results have not been included. This pig was diagnosed (Massey University Veterinary Clinic) as suffering from a bacterial meningitis which was not considered to be dietary related.

The pancreases were difficult to remove accurately being embedded in fat and mesentery. The stomach mucosa was also difficult to remove, being very thick and tough, while the small intestinal mucosa was easily removed, but increasingly difficult toward the terminal ileum. The mean relative organ weights of pigs fed either diet A or diet B are given in Table 5.2. Pigs receiving diet B compared to diet A had significantly ($P < 0.05$) lighter pancreases and a significantly ($P < 0.05$) heavier intestinal mucosa per unit total small intestinal weight. The latter, however, was not significantly heavier when expressed relative to body weight. Furthermore, the slightly higher proportions of mucosa in individual sections of the small intestine was not significant from pigs fed diet B compared to diet A. All other relative organ weights were similar between the diets. However, the kidney, liver, caecum and large intestine tended to be heavier and the stomach and small intestine lighter for the pigs fed diet B, though these effects were not statistically significant.

Mean pH and weights of the stomach and caecal contents, the activities of proteolytic enzymes (pepsin, trypsin, chymotrypsin and aminopeptidase N) and protein concentrations at the terminal ileum and in pancreatic tissue for pigs fed either diet A or diet B are reported in Table 5.3. The weight of the stomach contents was significantly ($P < 0.05$) less, aminopeptidase N activity (expressed as enzymatic units (U)/g mucosa and as U/100 g body weight) appeared ($P < 0.1$) higher and the total protein content of the ileal digesta tended ($P < 0.1$) to be higher in pigs fed diet B compared to those pigs fed diet A.

The activities of enzymes (alanine transaminase, aspartate transaminase and γ-glutamyl transpeptidase) in the jugular serum, and serum glucose, blood urea nitrogen (BUN),

creatinine and the BUN/creatinine ratio in pigs fed diets A or B are reported in Table 5.4. Significant differences were not detected between diets for these measures.

The total volume, total solids, total nitrogen, specific gravity, osmolality, urea nitrogen excretion, creatinine excretion and the urea N/creatinine ratios in pig's urine are given in Table 5.5. Urea N and total nitrogen appeared ($P < 0.1$) to be excreted at higher levels for pigs given diet B than for those on diet A. Although significant differences were not observed for any of the other measurements, it is noteworthy that the total solids, specific gravity, osmolality, creatinine and the urea N/creatinine ratio were all slightly higher in the urine of pigs fed diet B.

It was noticed that the urines from the pigs were different colours, and this appeared to be related to the diet which they had received. This effect is illustrated in Figure 5.1 (I). Diet A gave a normal straw-yellow coloured urine, whereas diet B gave a reddy-brown urine. The absorbance spectrums between 340 and 600 nm, identified the reddy-brown pigmentation to have a strong absorbance between 450 and 500 nm, Figure 5.1 (II).

Further urine analysis tests were carried out to attempt to identify the nature of these pigments: dipstick tests for bilirubin and urobilinogen; Erlich's test for hexosamines; a fluorescence test for porphyrins; indicans test; alkaptoneuria test; and iron content. The iron content was in similar concentrations in the urine from pigs fed diet A (0.443 ± 0.032 (SE) ppm) and diet B (0.513 ± 0.023 (SE) ppm). All of the above tests did not produced a positive response in the urine from pigs fed diet B.

Table 5.2 [1]Statistical level of significance: **NS** = not significant; * ($P < 0.05$). [2]Relative organ weights are wet tissue weights/100 g of final liveweight. [3]Mucosa samples are expresses per 100 g of the repsective digestive organ or small intestinal section.

Table 5.2 *Mean final liveweights, organ weights (expressed relative to final liveweight) and proportions of the mucosa weights (expressed relative to the respective organ weight) for pigs given the non-heated diet (A) n = 4, or the heated diet (B) n =3.*

	Diet		Overall standard error	Level of significance[1]
	A	B		
Final liveweight (kg)	19.0	19.2	0.8	NS
Relative organ weights (g/100 g body weight)[2]:				
Pancreas	0.227	0.195	0.005	*
Kidney	0.316	0.324	0.022	NS
Liver	2.370	2.410	0.040	NS
Stomach:	0.878	0.737	0.057	NS
mucosa	0.385	0.300	0.040	NS
Small intestine:	2.890	2.790	0.120	NS
mucosa	1.520	1.550	0.070	NS
Caecum	0.168	0.177	0.012	NS
Large intestine	1.040	1.150	0.050	NS
Mucosa:				
Stomach mucosa:	43.8	40.7	1.0	NS
Small intestinal mucosa:	52.6	55.6	0.5	*
section 1	56.2	58.4	1.6	NS
2	60.2	62.8	0.9	NS
3	59.7	63.2	0.9	NS
4	60.5	59.6	1.3	NS
5	51.5	52.1	0.9	NS
6	39.5	40.9	1.7	NS

Table 5.3 [1]Statistical level of significance: **NS** = not significant; **NS**$^{+}$ = ($P < 0.1$). [2]Proteolytic enzyme assays are reported in Chapter 2 - 2.8.7. Activities were measured with the following units: (i) 1 unit of activity = hydrolysis of 1µmol of substrate/min. Substrates for enzymes were - aminopeptidase N (L-alanine-4-*p*-nitoanilide); chymotrypsin (N-succinyl-L-phenylalanyl-4-*p*-nitroanalide); trypsin (N-benzoyl-DL-arginine- 4-*p*-nitroanalide). (ii) Pepsin activity was expressed as a change in absorbance at 280 nm/min from the hydrolysis of haemoglobin. [4]Freeze-dried weight. [4]Expressed per 100 g of body weight (**BW**).

Table 5.3 *Mean pH and weights of the stomach and caecal contents, proteolytic enzyme activities (pepsin in the stomach, aminopeptidase N in the mucosa of the small intestine, and chymotrypsin and trypsin in the pancreas and terminal ileal digesta) and protein concentrations (in the terminal ileal digesta and in pancreatic tissue) for pigs fed either the non-heated diet (A) n = 4, or the heated diet (B) n =3.*

	Diet		Overall standard error	Level of significance[1]
	A	B		
Stomach contents:				
weight (g)	600.00	424.00	26.00	*
pH	3.88	3.65	0.23	NS
Caecal contents:				
weight (g)	71.60	86.20	11.80	NS
pH	6.45	6.41	0.05	NS
Proteolytic enzyme activities[2]:				
STOMACH				
Pepsin				
Contents:				
(ΔA min^{-1} g^{-1} dry matter[3])	3.77	3.15	1.09	NS
(ΔA min^{-1} 100 g^{-1} BW[4])	2.91	1.38	0.76	NS
Mucosa:				
(ΔA min^{-1} g^{-1} tissue)	109.00	109.00	8.00	NS
(ΔA min^{-1}100 g^{-1} BW)	38.00	32.30	3.40	NS
SMALL INTESTINE				
Aminopeptidase N:				
(U/g mucosa)	2.14	3.07	0.19	NS+
(U/100 g BW)	3.18	4.79	0.34	NS+
Chymotrypsin:				
Ileal contents (U/g dry)	0.13	0.11	0.01	NS
Pancreas (U/g tissue)	1.27	1.41	0.13	NS
(U/100 g BW)	0.290	0.275	0.030	NS
Trypsin:				
Ileal contents (U/g dry)	3.04	2.10	0.43	NS
Pancreas (U/g tissue)	94.2	100.3	5.2	NS
(U/100 g BW)	21.3	19.5	1.1	NS
Protein concentrations:				
Ileal contents (mg/g dry)	68.5	78.2	3.5	NS+
Pancreas (mg/g tissue)	164.5	167.6	4.1	NS

Table 5.4 [1]Statistical level of significance: NS = not significant. [2]Enzymate assays are reported in Chapter 2 - 2.8.7, with 1unit of activity = the hydrolysis of 1 μmol of substrate/min: Substrates are - transaminases (NADH) and γ-glutamyl transpeptidase (L-γ-glutamyl-3-carboxy-4-*p*-nitroanalide).

Table 5.5 [1]Statistical level of significance: NS = not significant.

Table 5.4 *Mean jugular serum enzymatic activities of alanine transaminase (ALT), aspartate transaminase (AST), γ-glutamyl transpeptidase (γ-GT) activities; concentrations of glucose, blood urea nitrogen (BUN) and creatinine; and the BUN/creatinine ratio, in pigs fed either the non-heated diet (A) n = 4, or the heated diet (B) n = 3.*

	Diet		Overall	Level of
	A	B	standard error	significance[1]
Enzymatic activities[2]:				
ALT (U/L)	25.78	24.70	1.41	NS
AST (U/L)	27.77	27.73	3.32	NS
γ-GT (U/L)	70.70	50.20	7.00	NS
Glucose (g/L)	1.19	1.16	0.11	NS
BUN (mM)	6.46	7.53	0.45	NS
Creatinine (mM)	79.75	71.00	4.78	NS
BUN/Creatinine	10.14	13.10	0.94	NS

Table 5.5 *Mean urine daily excretion volumes; excretion of total solids, total nitrogen (N), osmolality, urea nitrogen (N) and creatinine; and the urea N/ creatinine ratio and specific gravity in the urine, from pigs fed either the non-heated diet (A) n =4, or the heated diet (B) n =3*

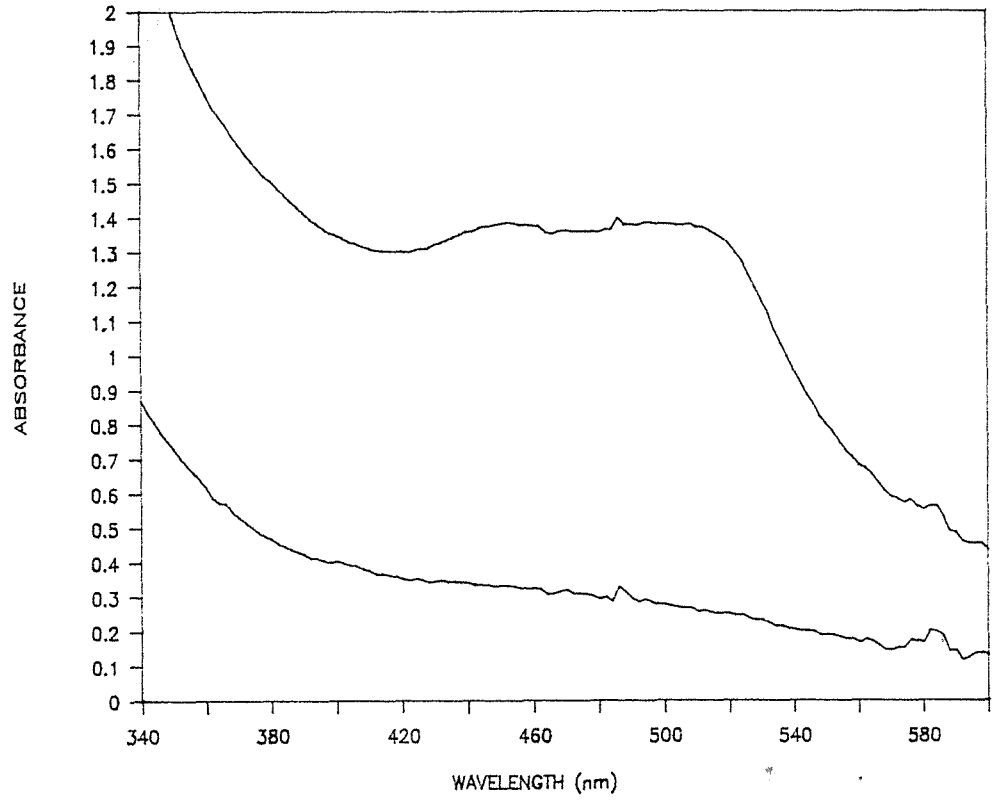
	Diet		Overall standard	Level of
	A	B	error	significance[1]
Excretion per $kg^{0.75}$ per day:				
Volume (ml)	144	135	10	NS
Total solids (g)	2.35	3.15	0.26	NS
Total N (g)	0.613	0.745	0.045	NS+
Osmolality	130	179	24	NS
Urea N (g)	0.490	0.631	0.035	NS+
Creatinine (g)	0.064	0.068	0.004	NS
Urea N/Creatinine	7.81	9.43	0.36	NS
Specific gravity	1.0045	1.0060	0.0010	NS

Figure 5.1 **(I)** From left to right: the diet fed to the corresponding pigs were pig #1 B, #2 A, #3 B, #4 A, #5 B, #6 A, #7 A and # 8 B. Pigs receiving the non-heated diet (A) have a normal straw-yellow urine colour whereas pigs recieving the heated diet (B) have a reddy-brown colour. Previous analysis (refer Chapter 4 discussion) has shown that the pigmentation is unlikely to be due to the brown Maillard pigments (refer Capter 4). **(II)** Undiluted urine absorbances.

Figure 5.1 *(I) Colour and (II) absorbance spectrums between 340 and 600 nm of the urine from pigs fed either the non-heated diet (A) or heated diet (B).*

(I)

(II)



5.3 Discussion

Pigs fed either the non-heated or heated diet consumed their respective diets readily, with total food intakes being similar over the 26 days of feeding. Although this is a relatively short time period, this indicates that the pigs were in a nutritionally similar state and that the ingestion of the early Maillard browned casein had no direct effect over this period. Incidents of reduced food intake and reduced weight gain appear to be a common feature of animals fed Maillard products (Lee *et al.*, 1981; and O'Brein & Walker, 1988). The period of sampling used in the present study (26 days) may have been too short to detect such differences in growth performance as they have been reported (Kimiagar *et al.*, 1980) to become more apparent over prolonged feeding Maillard browned egg albumin in nutritional similar diets.

There was no incidence of diarrhoea from the ingestion of the early Maillard browned protein. Similarly, diarrhoea was not encountered in rats fed browned egg albumin (Tanaka *et al.*, 1977; Kimiagar *et al.*, 1980; and Pintauro *et al.*, 1983) and appears only to be noted after giving diets with extremely high concentrations of either Maillard browned products, typically from the reaction between free amino acids and reducing sugars (Lee *et al.*, 1976; Adrian, 1974; and O'Brein & Walker, 1988), or from severely heat-damaged pure proteins (Percival & Schneeman, 1979). It has also been reported (Lee & Chichester, 1983) that ingestion of high levels of Maillard compounds may lead to a decrease in the rate of stomach emptying and to an enlarged caecum (Lee & Chichester, 1983). The caecum of the pigs in the present study was not enlarged due to feeding the heated diet and the pH of its contents were also similar.

The variability of the relative organ weights was generally low (coefficients of variation CV < 10 %). The similarity between diets of the relative organ weights (stomach, small intestine, caecum, large intestine, liver and kidneys) indicated that their growth was not affected by the ingestion of the heated diet after 26 days; it is notable that over this time period the pigs body weight had doubled. The pancreas was significantly lighter in pigs fed the heated diet, which is in contrast to the findings of Percival and Schneeman (1979) and Schneeman and Dunaif (1984) with growing rats, where no effect was found. However, it is of interest from an intravenous injection of ε-deoxyfructosyl-U^{14}C-lysine

into the tail vein of rats (Finot & Magnenat, 1981), that the whole body autoradioagram showed radioactivity mainly localized in not only the kidneys (medular zone), salivary and Harder's gland, but also the pancreas. These findings may hold some significance to the present results.

The pepsin activities in the stomach contents were highly variable (CV > 50 %) between individual measurements when expressed on a dry matter basis or related to final liveweight. Thus, and especially with view to the low numbers of animals per diet, it is inconclusive as to whether real differences exist. Pepsin activities in the mucosa wall were less variable (CV < 20 %), but no effect of diet was detected. The relative weight of the stomach and the pH of its contents were similar between the diets. However, the rate of stomach emptying appeared to be increased as a result of feeding the heated diet (refer Chapter 4). This may be a result of the consistency of the stomach contents (refer discussion, Chapter 4).

Trypsin activities were much higher than chymotrypsin activities in the present study, with the trypsin estimates being similar to values reported by other workers (Nitsan & Leiner, 1976; Glazer & Steer, 1977; and Schneeman & Dunaif, 1984). The latter found similar activities of chymotrypsin and trypsin in the pancreas and small intestine of rats. However, different substrates (either N-benzoyl-L-tyrosine ethyl ester, or N-glutaryl-L-phenylalanine-*p*-nitroanalide) were used in these chymotrypsin assays to that used in the present investigation (N-succinyl-L-phenylalanine-*p*-nitroanalide). This may explain why the present activities are lower. The activities of pancreatic or digesta chymotrypsin and trypsin were not affected by the ingestion of early Maillard browned casein, this indicated no effect on the protein digestive system of the small intestine. There appeared, however, to be a higher content of protein in digesta at the terminal ileum for pigs fed the heated diet. Percival & Schneeman (1979) and Schneeman & Dunaif (1984) found increased activities of trypsin and chymotrypsin and an increased digesta protein content in the small intestine of rats fed severely heat damaged pure casein and Maillard damaged dried milk respectively. The apparent lack of adaptation of these enzymes to the heated diet, in the present study, may be because of the nutritional adequacy of the protein fed to the pigs.

Since weight of mucosa in the small intestine was significantly increased and aminopeptidase N activity appeared to be elevated due to feeding the heated diet, adaptation of the small intestine to processing poorly digestible material may have

occurred. Aminopeptidase N constitutes 5 - 15 % of the total membrane protein and is the major membrane peptidase responsible for the hydrolysis of oligopeptides in the mucosa to di and tripeptides for subsequent absorption into the epithelial cells (Oste *et al.*, 1986). It has been shown that the brush border uptake is the rate limiting step in the absorption of certain tetrapeptides (Abidi and Morse, 1977). Elevation of the activity of this key enzyme could be a result of its inhibition by Maillard products, as reported by Oste *et al.* (1986). This, together with the notable increase in mucosal weight, appears to be an adaptive response to the greater proportions of oligopeptides formed due to heat treatment (refer Chapter 4).

Liver responses to the metabolic transit of Maillard products by increases in the jugular serum activities of alanine transaminase, aspartate transaminase and γ-glutamyl transpeptidase were not found. The determined levels are similar to the normal levels in human jugular serum (Teitz, 1986), with the transaminases being between 15 - 30 U/ml and transpeptidase being ≤ 50 U/L. Previous reports (Tanaka *et al.*, 1977 and Kimiagar *et al.*, 1980) have shown an increase in the activities of alkaline phosphatase and aspartate transaminase, as well as liver enlargement, in response to feeding heat-damaged proteins to rats over 3 months, implying physiological damage to the liver. From the present results there appears to be no physiological damage to the liver after the short term feeding of the heated diet to pigs.

Since the blood urea nitrogen and creatinine concentrations in jugular serum and their ratio were similar between the two diets, the kidney (and liver) functionings seem unaffected by the metabolic transit and presence of Maillard products. These values are also similar to the normal ranges in human serum (Eastham, 1978; and Teitz, 1986) with blood urea nitrogen (BUN) between 2.8 - 6.7 mM; creatinine (C) 35 - 106 mM; and BUN/C ratio 12 - 16. An increase in the blood urea nitrogen was reported by Schneeman and Dunaif (1984) and suggested to be reflective of the poor availability of dietary protein. Enlargement of the kidneys in response to feeding heat damaged protein has also been demonstrated (Schneeman and Dunaif, 1984, Kimiagar *et al.*, 1980) and suggested to be due to a need to adapt to excrete nitrogenous compounds being absorbed but not utilized (Nesheim & Carpenter, 1967; Lee *et al.*, 1977 B; and Varnish & Carpenter, 1975). This was not the case in the present investigation.

In this study urinary creatinine excretions (66 mg $(kg^{0.75})^{-1}$ day^{-1}) were higher than values for pigs given skim milk power diets (43 mg $(kg^{0.75})^{-1}$ day^{-1}) reported by Moughan *et al.* (1989 II), but lower than estimates (Moughan & Smith, 1984) from pigs fed casein diets (105 mg $(kg^{0.75})^{-1}$ day^{-1}). The daily urinary urea nitrogen excretion of the present investigation (613 mg $(kg^{0.75})^{-1}$ day^{-1}) was much higher than values for pigs fed an enzymatically hydrolysed casein diet (93 mg$(kg^{0.75})^{-1}$ day^{-1}) with an ideal amino acid balance (Moughan & Smith, 1984). This is a result of feeding dietary protein at a very high dietary concentration, therefore, resulting in metabolism excessive amino acid catabolism. Consequently, the total nitrogen and urea nitrogen/creatinine ratios are higher than expected for pigs having a normal level of protein in the diet. Urinary total nitrogen, total solids, specific gravity and osmolality tended to be higher consequent upon feeding the heated diet, demonstrating that more compounds were being processed and excreted from the heated diet through the kidneys. However, no adaptation of the kidneys to the excretion of these compounds was detected.

The brown pigments excreted in the urine from pigs fed the heated diet did not respond positively to any of the urine analysis tests to determine its nature. The spectral data show that the pigments absorbed strongly between 450 and 500 nm. All forms of haemoglobin (oxyhaemoglobin, reduced haemoglobin, carboxyhaemoglobin, methaemoglobin, sulfhaemoglobin and cyanmethaemoglobin) have absorption maxima at 550 nm or above (Allen & Henry, 1974). An acid urine has been stated to darken on standing because of the formation of methaemoglobin if haemoglobin is present (Bradley & Benson, 1974). Since there was no change in the iron content in the urine it seems unlikely to be haemoglobin or one of the above derivatives. Other rarer cases of dark brown urine are the presence of homogentisic acid (alcaptoneuria) and melanin, which also darken on standing, furthermore, the presence of hexosamines and indicans also darkens urine (Bradley & Benson, 1974). Urine analysis identification for these compounds also resulted in negative results. A further possibility is breakdown products of haemoglobin which may occur under the acid conditions of the preserved urine, to cyclic (porphyrins) or linear tetrapyroles (bilirubin). Tests for the presence of these compounds were also negative. However, bilirubin and porphyrins may degrade on standing in acid conditions. From the absorbance spectrums the plateau may correspond to some form of melanoidins. Other studies, feeding browned egg albumin to rats for long periods (Kimiagar *et al.*, 1980; and Pintauro *et al.*, 1983) have not encountered discoloured urine. As discussed previously in detail (refer Chapter 4) it seems unlikely that these

pigments are from dietary melanoidins. Unfortunately, an acidified urine may cause change to the components present. Therefore the present results are inconclusive and require further investigation to determine the nature and significance of these pigments

In summary, when young growing pigs were fed a lysine supplemented early Maillard browned casein, which could be considered nutritionally adequate, at a relatively high level (240 g/kg (24 %) of heated early Maillard browned casein at a level of 8 % metabolic body weight) for 26 days, there were no major physiological effects detected. However, pigs receiving the heated diet had greater amounts of mucosa per unit weight of small intestine and aminopeptidase N activity appeared to be elevated. Furthermore, the latter animals had lighter pancreases and some differences in urine composition were noted. A difference of possible significance, was that the urine of the pigs receiving diet B was darker in colour in comparison to animals on diet A. No explanation could be found for this observation, but this merits further investigation.

Chapter 6

Overview

The information derived from the foregoing experiments adds to and generally supports the current ideas on nutritional and physiological responses, to the short-term ingestion of Maillard products. In the following overview the consequence of Maillard reactions in light of the present evidence are discussed with reference to future directives.

In Chapter 3, it was assumed that a balanced proportion of early and advanced Maillard products was reflective of the overall proportions in the human diet. Chemical analyses showed that mainly lysine was lost. Furthermore, acid hydrolysed lysine overestimated FDNB reactive lysine, since regenerated lysine was produced from the acid hydrolysis of ε-DF-L. Estimation of the ε-DF-L content in the heated mixtures distinguished the extent of the Maillard reaction, showing approximately equal proportions of early (ε-DF-L) and advanced Maillard products. The incorporation of $U^{14}C$-glucose was useful for labelling Maillard compounds, and together with an assesment of the ε-DF-L content, in the distinction of advanced Maillard products (residual radioactivity) from the total radioactivity present in samples, both *in-vitro* (Chapter 3) and *in-vivo* (Chapter 4). However, it was uncertain whether the residual radioactivity was totally reflective of advanced Maillard products, since results (see Chapter 4) indicated the possibility of additional $U^{14}C$-glucose, or its decomposition products, being adsorbed onto the protein. Further information on the effects of the formed Maillard products may arise from the analysis of the structural relationships of protein bound Maillard compounds. One such technique is *in-vitro* enzymatic digestion, commonly used as an assessment of the *in-vivo* availability of amino acids (Sgarbieri *et al.*, 1973; Carpenter & Booth, 1973; and Plakas *et al.*, 1985 & 1988). However, only few studies (Clark & Tannenbaum, 1970 & 1973; and Moller 1977 I, II & III) have used it to characterize the extent of limit peptide formation and for subsequent analysis of the digested protein bound Maillard compounds.

It was demonstrated that the acid hydrolysed products of ε-DF-L (pyridosine, regenerated lysine and furosine) varied considerably in their recoveries depending on the hydrolysis

conditions used (see Chapter 3). This is of particular concern to analyses using the equations from Finot *et al.*, (1981) under altered acid hydrolysis conditions and, further, to whether the difference in the behaviour of protein bound ε-DF-L compared with free ε-DF-L are similar under the latter conditions. The behaviour of ε-DF-L may vary depending on the type of physiological sample. Different acid hydrolysis conditions may be required for its total recovery in different physiological samples such as digesta, faeces, blood, tissues and urine. It may prove useful to analyze physiological samples using physiological ion-exchange chromatography, as this technique does not involve acid hydrolysis. These areas remain to be clearly investigated.

It was shown that the Maillard products in the early Maillard browned casein significantly decreased the ileal digestibility of nitrogen, and several non-essential and essential amino acids (see Chapter 4). However, the reduction was generally small (< 5 % units) with only lysine and arginine considered to be of practical significance. Additionally, ileal digestibility of lysine was influenced by the regenerated lysine from the acid hydrolysis of ε-DF-L. However, even though regenerated lysine from the hydrolysis of ε-DF-L greatly influenced acid hydrolysis lysine values in both the diet and ileal digesta, these measurements proved adequate in predicting the actual lysine availability in the early Maillard browned casein. This may also be similar in other food sources, but remains to be clearly investigated in practice.

It was shown from Chapter 4 that faecal lysine digestibility estimates were inadequate for measurements of apparent availability, being overestimates from the influence of the large intestinal microorganisms. This raises concern to the predictability of *in-vitro* methods of availability since FDNB available lysine (and others) are commonly compared with estimates of faecal digestibility (Bujard *et al.*, 1967 I & II; Palamidis & Markakis, 1980; Bookwalter & Kwolek, 1981; Craig & Broderick, 1981; Bjorck *et al.*, 1983; and Erbersdobler & Anderson, 1983). In these situations faecal digestible lysine has been shown to be higher than FDNB available lysine. The most predictive *in-vitro* chemical estimates of nutritionally available lysine in the presence of early Maillard products, are the direct FDNB, total lysine after borohydride treatment, guanidation and - in the specific case of milk powders - the furosine technique (Hurrell & Carpenter, 1981). All of these methods do not account for a decreased *in-vivo* digestibility of lysine; which may not be detected by faecal digestibility estimates. In these situations it would be more predictive to use *in-vitro* techniques which account for digestibility, such as the enzymatic digestive

methods. There is, however, some contradiction to the predictability of enzymatic estimates on ileal availability. Taverner and Farrell (1981) show close relationships of ileal protein and lysine availability with nitrogen digestibility determined by an *in-vitro* pronase assay, on cereal grains fed to rats. In contrast, Moughan *et al.* (1989 II) using a multi-enzyme digestibility assay and the pronase assay reported that these did not correlate with the *in-vivo* digestibility of crude protein in meat and bone meals fed to rats. The validity of method appears to vary for different food sources, possibly being influenced by other compositional factors. Plakas *et al.* (1988) has demonstrated *in-vitro* enzymatic hydrolysis to be a predictive measure of *in-vivo* lysine bioavailability (by plasma measures) of early Maillard browned protein. These techniques merit further investigation.

In Chapter 4, the reduction in the availability of essential amino acids appeared to be accounted for by the presence of small limit peptides which may contain Maillard products. The contribution from absorbed but unavailable amino acids (which were finally excreted in the urine) was negligible. Further emphasis should be directed toward the analysis of digesta at the terminal ileum, particularly on the size distribution and properties in relation to the digesta composition. Evidence in Chapter 4 has also contributed to the need for further analysis of the effect of Maillard products on the absorption of other oligopeptides and free amino acids. Recent developments in this area (Erbersdobler, 1981; and Sherr *et al.*, 1989) have documented influences of free Maillard products on the absorption at the gut wall, but the magnitude, mechanisms and significance of these remain unanswered, particularly in relation to protein bound Maillard products. Furthermore, the apparent ileal digestibility measurement of absorption, and the actual amounts entering the portal blood stream, may be dissimilar because of the metabolic interactions of the gut wall barrier. It is of interest that in Chapter 5, the protein digestive functioning and adaptation of the gastrointestinal tract remained virtually unchanged, except for a small response at the small intestinal mucosa by the apparent increases in aminopeptidase N activity and proportions of mucosa. Adaptation of the gastrointestinal tract would seem likely in growth and development from the presence of absorbed non-utilizable compounds. However, the long-term effects ($\geq$ 12 months) of their presence and the significance, in nutritionally balanced diets, is yet to be established. Analysis of these tissues based on histopathological examination would be advisable as recent studies (O'Brien & Walker, 1988) have documented changes in tissue integrity on feeding Maillard products to rats.

The metabolic transit of Maillard products, from that absorbed by the terminal ileum to the final excretion in the urine was determined. Present results (see Chapter 4) indicated that only a small portion of the absorbed Maillard compounds were excreted in the urine. This is contrary to other experimental evidence of virtually complete excretion of Maillard products administered intravenously in rats (Erbersdobler, 1981; Finot & Magnenat, 1981; and Sherr *et al.*, 1989). The influence of metabolic transit on passage through the gut wall may account for these differences. The liver and kidneys have previously been shown to transport fructose amino acids (Erbersdobler, 1981), with both ε-DF-L and ε,α-diDF-L shown to be present in liver microsomes (Sherr *et al.*, 1989), whereas the transport across skeletal muscle tissue was negligible (Erbersdobler, 1981). There appeared to be no physiological responses due to short-term feeding of early Maillard browned casein in pigs, except for of reddy-brown pigments of an unknown nature in present in the urine (see Chapter 5). The lack of physiological responses over short-term feeding does not indicate that changes are not occurring on a lower level, becoming apparent only after long-term feeding. Since histopathological changes have also been noted in the liver tissue (O'Brien & Walker, 1988) and particularly kidney tissue (Erbersdobler, 1981; and O'Brien & Walker, 1988), adaptation to the metabolic transit of Maillard products also seems likely at high enough levels of intake for long enough periods of feeding.

Overlying all of the concerns of Maillard reactions in relation to human nutrition, are uncertainties of the quantitative and qualitative aspects of Maillard compounds in the diet. Since many different heating techniques are applied to a variety of food sources only detailed and extensive nutritional surveys will determine the types and levels of Maillard compounds ingested. The preference to cooking techniques and food sources will be influenced by both the cultural and social structures.

Some concern is raised over inferences of antinutritive or inhibitory properties of ingested Maillard products, from the decrease in nutritive values not being completely restored by the loss or unavailability of amino acids (Rao *et al.*, 1963; and Sgarbieri *et al.*, 1973). Firstly, the supplementation of the limited essential amino acids was based on *in-vitro* losses, measured by amino acid and enzymatic analysis. Although the latter technique is probably the most accurate *in-vitro* assessment of availability (Plakas *et al.*, 1988), differences may exist with *in-vivo* availability, possibly underestimating the

supplementation needed. Secondly, criticism (Pintauro *et al.*, 1983) has been raised as to the use of free amino acids as supplements, since Rodgers and Harper (1965) reported that this was insufficient to support the growth rate of rats equal to that of the amino acids in intact proteins. However, in cases where browned and controlled proteins are fed at more than adequate levels and/or have the same protein efficiency ratios, can implications of the physiological activeness or inactiveness of the ingested Maillard products be reliably discussed.

The inference of toxicity of Maillard products must be considered in light of many factors, other than the physiological responses these appear to elicit. By definition a toxic compound is one which has a poisonous effect, damaging or impairing the activity of body tissues, being potentially lethal. Many compounds are "potentially" toxic, however, the following questions must be answered for a full analysis of the role of this toxicity (Fogleman, 1981).

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(1) What are the exposure conditions and what groups of people may it effect?

(2) What dosage levels cause the toxicity, and what are the social intake levels?

(3) What are the effects, both good and bad?

(4) Do the advantages outweigh the disadvantages (Benefit/Risk)?

Currently, the role of toxicity remains an open issue as levels of intake, the long-term physiological responses and their significance remain to be investigated in relation to human nutrition.

In conclusion from the present investigation feeding the early Maillard browned casein to the pig, an anatomically and physiologically similar model for humans, caused small changes in overall protein digestibility. Detrimental physiological responses were not apparent after short-term (26 day) feeding, in a nutritionally adequate diet.

Appendix 4.1 [1]Calculation of digestibility either by reference to an indigestible marker (chromic oxide) or by total collection over a 5 day period. [2]Statistical significance: **NS** = not significant; * ($P < 0.05$); ** ($P < 0.01$) and *** ($P < 0.001$).

Appendix 4.1

The mean apparent faecal digestibility of amino acids and nitrogen calculated by reference to the indigestible marker chromic oxide, or by total collection.

Amino acid	Method[1]		Overall	Level of
	Marker	Total collection	standard error	significance[2]
Aspartic acid	96.0	96.7	0.16	**
Threonine	96.5	97.3	0.13	***
Serine	97.1	97.8	0.14	**
Glutamic acid	98.4	98.7	0.08	**
Proline	90.1	93.7	0.78	***
Glycine	92.2	93.8	0.50	*
Alanine	94.1	95.6	0.25	**
Valine	97.6	97.8	0.52	NS
Methione	97.5	97.5	0.59	NS
Isoleucine	97.3	97.9	0.11	***
Leucine	97.5	98.1	0.09	***
Tyrosine	97.6	98.2	0.10	**
Phenylalanine	97.3	98.0	0.16	**
Histidine	97.6	98.1	0.11	**
Lysine	98.1	98.2	0.34	NS
Arginine	95.5	96.8	0.15	***
Nitrogen	96.7	97.4	0.38	NS

Appendix 4.2 [1] **Asp** = aspartic acid, **Thr** = threonine, **Ser** = serine, **Glu** = glutamic acid, **Pro** = proline, **Gly** = glycine, **Ala** = alanine, **Val** = valine, **Met** = methionine, **Ile** = isoleucine, **Leu** = leucine, **Tyr** = tyrosine, **Phe** = phenylalanine, **His** = histidine, **Lys** = lysine, and **Arg** = arginine. [2]Statistical significance: **NS** = not significant; * ($P < 0.05$); ** ($P < 0.01$) and *** ($P < 0.001$).

Appendix 4.3 [1] **Asp** = aspartic acid, **Thr** = threonine, **Ser** = serine, **Glu** = glutamic acid, **Pro** = proline, **Gly** = glycine, **Ala** = alanine, **Val** = valine, **Met** = methionine, **Ile** = isoleucine, **Leu** = leucine, **Tyr** = tyrosine, **Phe** = phenylalanine, **His** = histidine, **Lys** = lysine, and **Arg** = arginine. [2]Actual lysine estimates, determined by subtracting regenerated lysine (from the hydrolysis of ε-DF-L) from acid hydrolysis lysine. [3]Residual radioactivity calculated from subtracting the radioactivity attributed to ε-DF-L (3.33×10^9 dpm/mol) from the total radioactivity - 3.3.3.

Appendix 4.2

The overall means for the apparent digestibility of amino acids and nitrogen within diet (A or BR) or source (ileal, IL or faecal, F) and their interaction (diet x source).

AA[1]	Diet (D)		Source (S)		Level of significance[2]			Overall standard error
	A	BR	IL	F	D	S	DxS	
Asp	92.69	91.71	89.19	95.71	NS	***	NS	1.43
Thr	90.95	87.26	82.89	96.47	***	***	*	1.62
Ser	93.60	90.97	88.28	97.06	***	***	NS	0.92
Glu	96.85	95.33	94.18	98.39	***	***	*	0.45
Pro	97.91	96.24	95.41	99.10	***	***	*	0.38
Gly	71.20	70.26	51.98	92.23	NS	***	NS	11.90
Ala	92.15	89.94	88.55	94.06	NS	***	NS	2.03
Val	95.88	93.87	92.65	97.56	***	***	*	1.16
Met	98.25	96.17	97.08	97.51	***	NS	NS	0.65
Ile	96.55	94.59	94.20	97.27	***	***	NS	0.88
Leu	97.23	96.53	96.34	97.54	NS	*	NS	0.81
Tyr	97.58	96.54	96.61	97.64	*	*	NS	0.69
Phe	96.74	96.14	95.73	97.30	NS	***	NS	0.84
His	97.09	94.77	94.65	97.56	***	***	*	0.70
Lys	97.36	75.97[3]	78.27	98.11	***	***	***	1.43
Arg	95.50	90.76	88.28	97.06	***	***	***	1.50
Nit	93.76	89.01	87.04	96.70	***	***	*	1.14

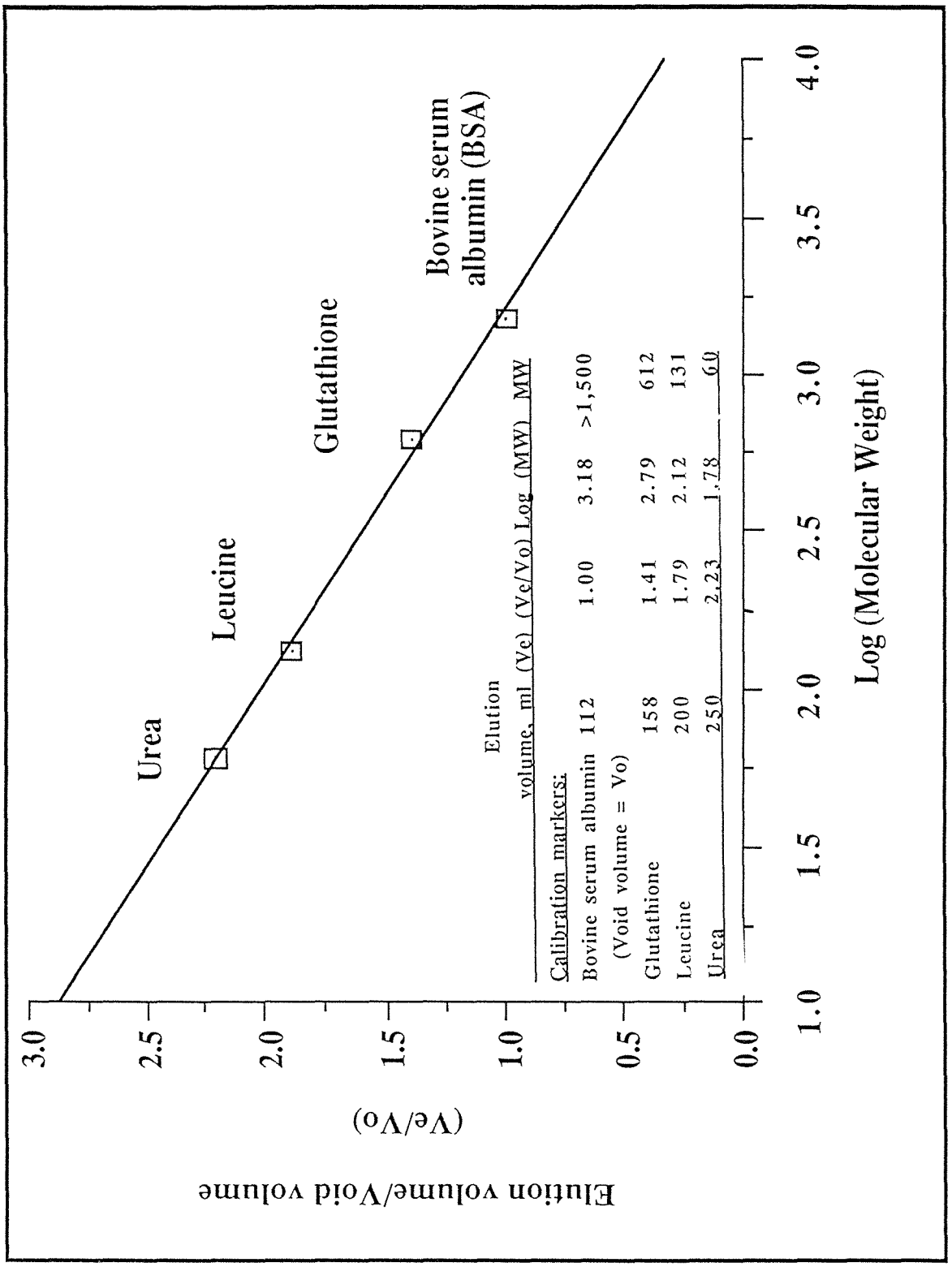
Appendix 4.3

Amino acid, nitrogen, dry matter, actual lysine, ε-DF-L, total radioactivity and residual radioactivity digestibility coefficients in sections of the small intestine, of the samples pooled across pigs according to diet (A or BR)

Amino acid	Small intestinal section							
	(3)		(4)		(5)		(6)	
	A	BR	A	BR	A	BR	A	BR
Aspartic acid	78	78	75	79	81	79	81	82
Threonine	79	68	75	68	80	70	77	72
Serine	82	78	82	78	88	79	85	81
Glutamic acid	87	83	87	83	91	88	81	80
Proline	92	81	92	85	93	79	93	90
Glycine	42	43	30	9	30	10	18	10
Alanine	79	82	73	81	79	78	79	83
Valine	88	85	86	87	87	82	89	88
Methionine	94	89	91	91	92	89	93	94
Isoleucine	88	83	86	84	90	84	90	90
Leucine	90	91	87	91	91	91	92	94
Tyrosine	92	92	90	94	92	93	93	94
Phenylalanine	90	86	98	93	90	91	93	93
Histidine	91	83	89	88	91	87	91	89
Lysine	92	58	88	34	91	37	94	3
Arginine	87	89	80	83	85	80	85	82
Average:	87	83	84	78	88	84	88	87
Nitrogen:	82	78	82	78	84	81	82	79
Dry matter:	78	78	80	80	82	80	83	82
Actual lysine[2]:	92	75	88	56	91	57	91	63
ε-DF-L:	NA	20	NA	-15	NA	-10	NA	-37
Radioactivity:								
Total	NA	59	NA	53	NA	41	NA	45
Residual[3]	NA	78	NA	76	NA	67	NA	80

Appendix 4.4

Molecular weight calibration curve of the Sephadex G-15 column used for the fractionation of ileal digesta and urine from pigs fed either the non-heated diet (A) or the heated diet (BR).



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