RESEARCH INTO CAUSES OF DIARRHOEA ASSOCIATED WITH THE HILL'S PRESCRIPTION DIET CANINE Z/D® ULTRA ALLERGEN FREE

A thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Animal Science at Massey University, Palmerston North, New Zealand

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Abstract

The history, manufacture, physical and immunological characteristics, nutritional adequacy, formulation, taste and digestibility of protein hydrolysates were reviewed. Studies were then undertaken to investigate the cause of the diarrhoea reported to occur in dogs fed an early formulation of Hill's Prescription Diet Canine z/d® ULTRA Allergen Free.

It was hypothesised that the digestibility of Canine z/d® ULTRA Allergen Free was poor and therefore contributing to the diarrhoea. To test this hypothesis a study was designed to assess the apparent ileal digestibility of the Canine z/d® ULTRA Allergen Free diet using a rat model of apparent ileal digestibility. The results of this study showed the apparent ileal digestibility of the Canine z/d® ULTRA Allergen Free diet was high and it was concluded that the digestibility of the diet does not predispose dogs to diarrhoea.

The reported high osmolarity of protein hydrolysate diets was considered a potential contributing cause to the diarrhoea. To test this hypothesis, the osmolarity of the Canine z/d® ULTRA Allergen Free diet was compared to a standard maintenance diet and to a diet formulated for the treatment of diarrhoea. The osmolarity of the Canine z/d® ULTRA Allergen Free diet was approximately twice that of the osmolarities of the other two diets. This difference was statistically significant ($p<0.05$), and the data from this study suggest that the hydrolysate diet was sufficiently hyperosmolar to be capable of damaging the mucosa. Although of interest, these observations do not, however, allow a conclusion that the reported diarrhoea is due to hyperosmolarity.

Lastly, breath hydrogen tests were performed to investigate whether carbohydrate malabsorption, abnormal orocolic transit times or small intestinal bacterial overgrowth played a role in the cause of the reported diarrhoea. The breath hydrogen concentrations of dogs fed the hydrolysate

diet remained within reference intervals confirming the high carbohydrate digestibility observed in the rat model of apparent ileal digestibility and providing no support for abnormal orocolic transit or bacterial overgrowth of the small intestine as causes for the reported diarrhoea.

In conclusion, the results of the studies in this thesis do not provide a clear explanation for the cause of the diarrhoea reported to occur with the Canine z/d® ULTRA Allergen Free diet.

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Chapter 1

Introduction

A connection between diet and "eczema" in dogs had already been hypothesised by the beginning of the 20th century. Although reports on the prevalence of food allergy vary, it is nowadays estimated that approximately 1% of all dermatological diseases in dogs and approximately 10% of all allergic skin reactions (except flea allergy) can be ascribed to food allergy (Ackerman, 1989; Muller *et al.*, 1989). Food sensitivity is an adverse response to an ingested food or food additive and can be due to a number of different causes (e.g. immunologic, pharmacologic, toxic, infectious, etc.). Food allergy and food hypersensitivity are synonymous terms and refer to an adverse reaction to a food or food additive with an immunological cause. In dogs, clinical signs of food allergy are usually dermatological or gastroenteric, or both (Roudebush *et al.*, 2000).

To establish a diagnosis of food allergy, it has been recommended to feed a restricted elimination diet for a minimum of three weeks (Muller *et al.*, 1989; Carlotti *et al*, 1990; White, 1986), but this recommendation is empirical and has not been based on any prospective studies (Rosser, 1993). Food allergies are most commonly due to allergic reactions to protein sources (Chandra, 1997). An elimination diet therefore consists of a protein and carbohydrate source to which the dog has had no previous exposure. Traditionally elimination diets are based on cooked lamb or chicken in combination with cooked rice. Veterinary diets are now available with a wide range of protein sources including chicken, fish, rabbit, duck, lamb and venison.

Unfortunately, it is possible for a patient to show allergic reactions to a wide range of protein sources (August, 1985). Multiple allergic patients can be successfully treated with diets based on protein hydrolysates (Hill *et al.*,

1995). Hydrolysis of protein reduces the protein to small peptides. Sufficiently small molecular fragments are not recognised by the recipient's immune system and are no longer allergenic (Cave, 2001).

Hill's Pet Nutrition (Topeka, Kansas) has recently launched Prescription Diet® Canine z/d® ULTRA Allergen Free, a diet based on hydrolysed chicken proteins. The diet is intended for the nutritional management of dogs with food allergy and intolerance. Unfortunately, the original formulation of this diet was associated with a higher than acceptable (to the company) number of reports of diarrhoea.

The research in this thesis will explore some potential causes by which the diet could predispose dogs to diarrhoea. In the second chapter, protein hydrolysates used in diets are reviewed. In Chapter 3, a rat model is used to assess the apparent ileal digestibility of Canine z/d® ULTRA Allergen Free as a poorly digestible diet can result in diarrhoea. High dietary osmolarity is another potential cause of diarrhoea particularly when feeding hydrolysate diets. In Chapter 4, the work in Chapter 3 was extended by comparing the osmolarity of Canine z/d® ULTRA Allergen Free to a standard maintenance diet and to a diet formulated for the treatment of diarrhoea. The final study of this thesis is described in Chapter 5. In this study, a breath hydrogen test was performed to assess if the Canine z/d® ULTRA Allergen Free diet was associated with carbohydrate malabsorption, small intestinal bacterial overgrowth and/or abnormal orocolic transit times, all of which predispose to diarrhoea. Chapter 6 states general conclusions drawn from the three different studies and gives some approaches by which the diarrhoea associated with the original formulation of Canine z/d® ULTRA Allergen Free could be further investigated.

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Chapter 2

A review of protein hydrolysates for use in food

2.1 Introduction

This review gives a general overview of proteins and protein hydrolysates and their use in preventing adverse reactions to food.

2.2 What are proteins?

Proteins are a large group of naturally occurring complex organic nitrogenous compounds. Each protein is built from a combination of amino acids (Figure 2.1 and Figure 2.2) that are joined together through peptide bonds (Figure 2.3). Twenty-two amino acids have been identified as vital for proper growth, development and maintenance of health. The body can synthesize thirteen of these, the non-essential amino acids, whereas the remaining nine have to be obtained from the diet and are therefore called essential. Protein is the major source of building material for muscles, blood, skin, hair, nails, and the internal organs.

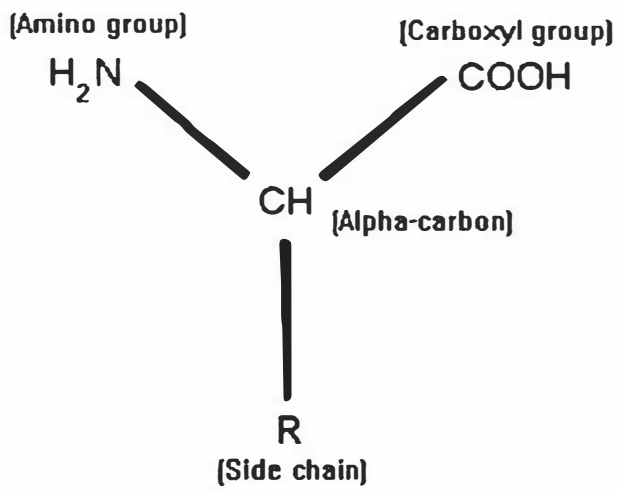

Figure 2.1 Primary structure of amino acid (modified from Friedli, 2000)

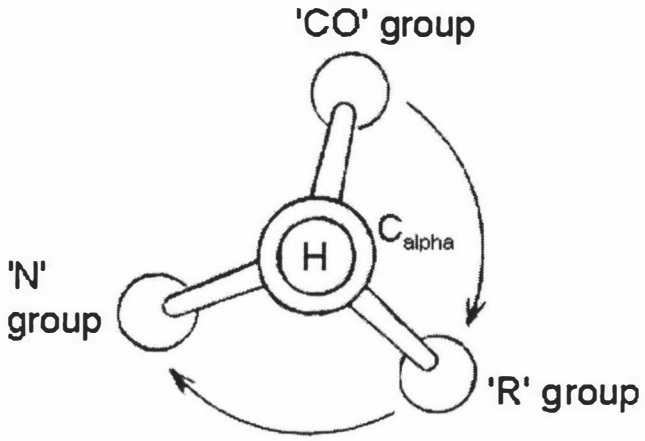

Figure 2.2 3-D structure of amino acid (modified from Friedli, 2000)

2.3 Protein hydrolysates and hydrolysis

The basis of the process of hydrolysis is the attacking of the peptide bonds (Figure 2.3) that hold the protein together (Figure 2.4). Hydrolysis is a chemical reaction in which large molecules are split into smaller molecules by reaction with water; often assisted by a catalyst. For example, in digestion, enzymes catalyse the hydrolysis of carbohydrates, proteins, and fats into smaller, soluble molecules. Hydrolysis changes the chemical, physical, biological, and immunological properties of proteins (Cordle, 1994). The protein is chopped into smaller pieces in a manner which is similar to that which happens to proteins that are ingested and exposed to digestive proteases. Protein hydrolysates can therefore be viewed as pre-digested proteins. A mix of single amino acids, dipeptides and tripeptides (Figure 2.3) may be considered the optimal form of pre-digested protein.

Over the past few years, some authors have shown that preparations rich in small peptides from partially hydrolysed proteins are utilized more efficiently and have a higher nutritive value than a similar mixture of free amino acids (Monchi and Rerat, 1993; Ziegler *et al.*, 1990; Morato *et al.*, 2000) (see section 2.14 for a discussion of the nutritive value of protein hydrolysates).

This is probably because these small peptides are more rapidly absorbed than the free amino acids.

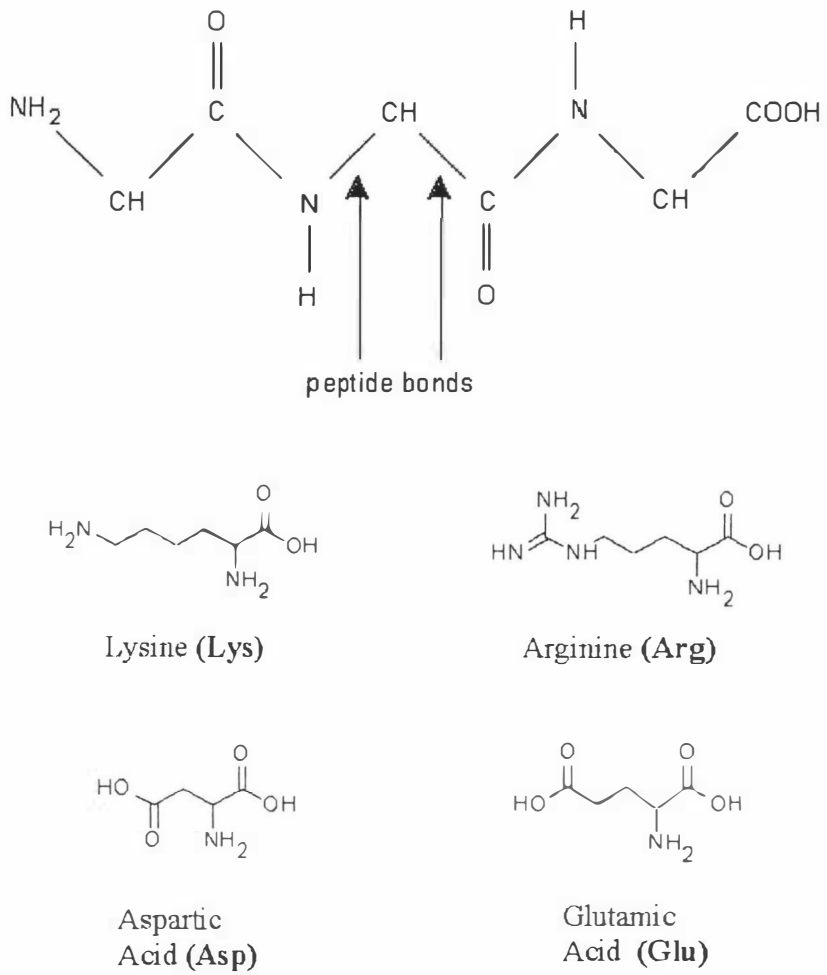

Figure 2.3 Example of peptide bonds and four different amino acids

2.4 Purpose of hydrolysis

Hydrolysis of proteins is carried out for many reasons. The most frequent purpose is to improve the functional and nutritional properties of proteins. Functional properties include adding texture to a food, improving the solubility, foaming and emulsification properties, removing aversive-flavours or improving flavours, accelerating the ripening of cheese, improving heat stability, retarding deterioration and removal of toxic or inhibitory ingredients (Lahl and Braun, 1994; Clemente, 2000).

One of the best-established clinical purposes of protein hydrolysis is to reduce the risk of allergenicity in human breast milk substitutes. Other clinical uses are treatment and management of food allergies, inflammatory bowel disease, short bowel syndrome, transient small bowel enteropathies due to food sensitivity, allergic colitis, cystic fibrosis and pancreatic insufficiency (Milla, 1991; Schmidl *et al.*, 1994).

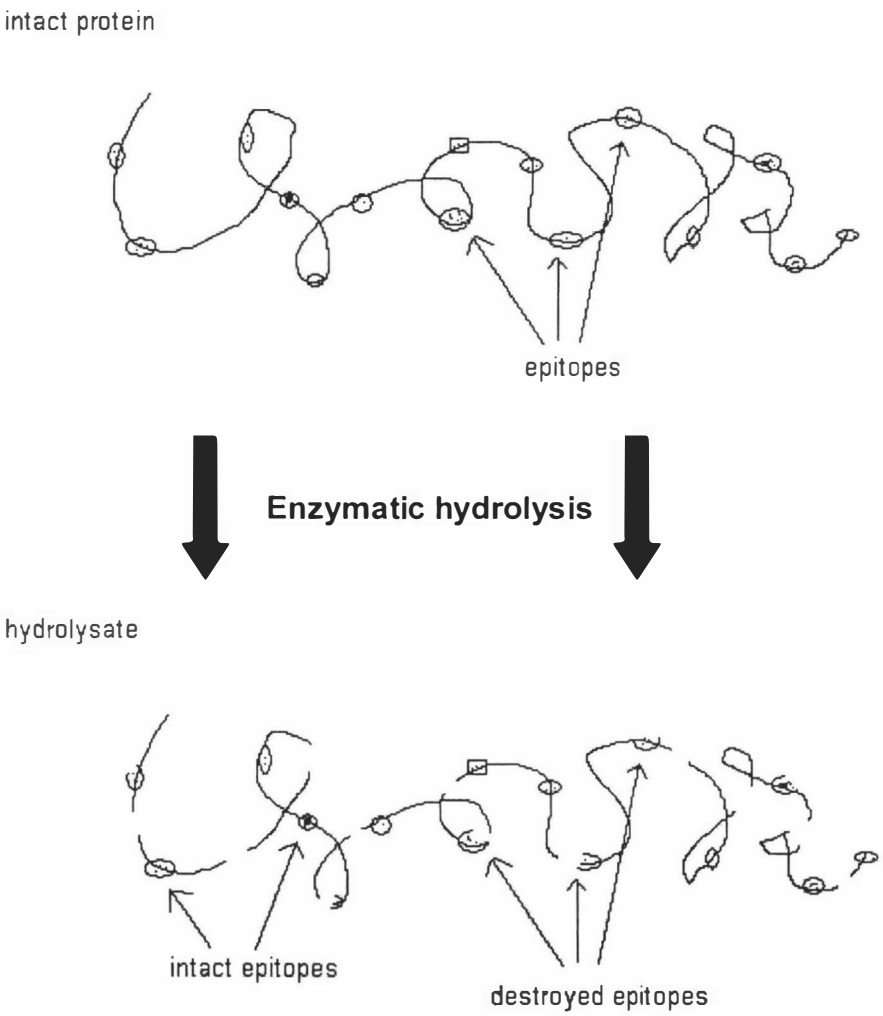

Figure 2.4 Schematic explanation of the destruction of epitopes of proteins during enzymatic hydrolysis (adapted from Sawatzki *et al.*, 1994)

2.5 History of protein hydrolysates

References to the use of enzymes from animal stomachs for cheese making are found in the Greek epic poems *The Iliad* and *The Odyssey* dating as far back as 700 B.C. (cited in Barfoed, 1981). However, soy sauce is considered to be the first protein hydrolysate (Dave *et al.*, 1991; Prendergast, 1974). For centuries this has been made by cooking soyabeans and wheat together and then leaving the mixture to stand for six months, during which time proteolytic enzymes break the peptide linkages of soy (Dave *et al.*, 1991).

A distinction has to be drawn between protein hydrolysates that are made by the action of enzymes and protein hydrolysates that are made by fermentation by micro-organisms. Fermentative changes can result in many other chemical reactions in addition to the hydrolysis of protein. Furthermore, the technology of protein hydrolysis has developed from different roots than the technology of fermented foods (Adler-Nissen, 1986a).

Braconnot performed the first experiments on the acid hydrolysis of proteins in 1820 (Hill, 1965) and the first protein hydrolysate of this kind was introduced as a flavouring agent for foods in 1886. This was an acid hydrolysed vegetable protein (HVP) that is nowadays used extensively as a flavouring agent by the food industry. The first commercially-available protein hydrolysates for use as food appeared during World War II (Adler-Nissen, 1986a; Cordano and Cook, 1985 cited in Mahmoud, 1994). A pepsin-modified soya protein was used to partly replace egg white in the confectionary industry and a casein hydrolysate was used for feeding low birth weight infants.

Since the 1940's considerable attention has been given to the use of amino acids and protein hydrolysates for feeding patients who cannot digest intact protein (Cuthbertson, 1950). At present, the treatment of food allergy is one of the principal driving forces behind the development of hydrolysate technology (Cordle, 1994).

2.6 Manufacture of protein hydrolysates

Protein hydrolysates can be made with enzymes, acids or alkalis. Because of their specificity, enzymes are strongly preferred over acids or alkalis for hydrolysis of proteins to be used in nutritional applications (Lahl and Braun, 1994; Clemente, 2000). Furthermore, enzymatic hydrolysis is developed under mild conditions of pH (6-8) and temperature (40-60°), avoiding the extremes usually required for chemical and physical treatments and minimising side reactions (Clemente, 2000). The ability of an enzyme to hydrolyse to the desired extent is usually the criterion for its selection in the manufacturing process. The extent of the hydrolysis of proteins is usually measured by the 'degree of hydrolysis' (DH). The DH is the ratio of free amino acids to total amino acids. This is expressed as the amount of amino nitrogen present in the hydrolysate relative to the total amount of nitrogen present in the substrate, which can be determined by formaldehyde titration and the Kjeldahl method (Silvestre, 1997).

The production of safe, effective hypoallergenic diets on a commercial scale has become of increasing importance (Hudson, 1995). Commercial-scale hydrolysis happens in batch processes, using large vessels containing watery mixtures of substrate and enzyme (Figure 2.5).

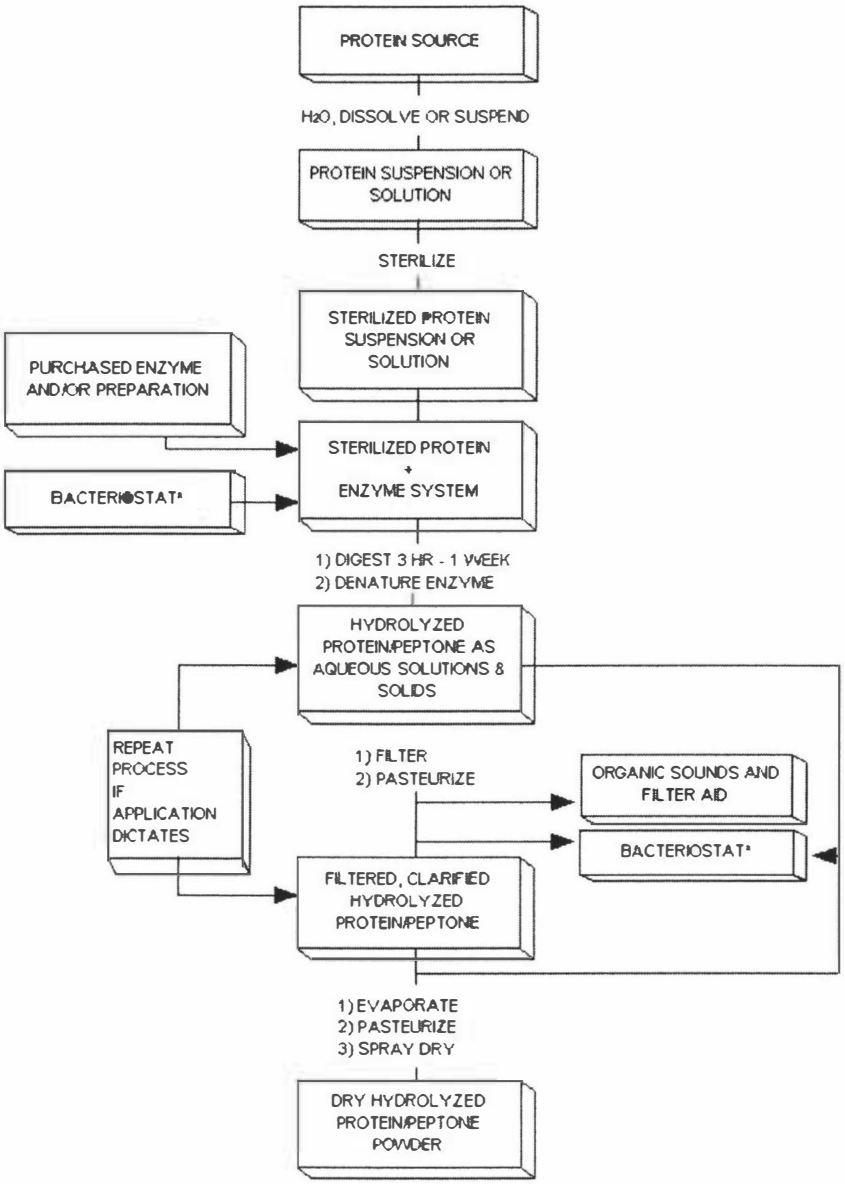

Figure 2.5 Manufacture of protein hydrolysates for nutritional products (from Lahl and Braun, 1994)

The preferred method for stopping the hydrolysis process is to adjust the pH and temperature to a level at which the enzyme activity is destroyed (Lahl and Braun, 1994). Another method to terminate the hydrolysis is removal of the enzyme by filtration of the mixture.

2.7 Substrates and enzymes

An enzyme is a protein with catalytic properties due to its power of specific activation (Dixon and Webb, 1979). Some enzymes that are commonly used in protein hydrolysis are trypsin, chymotrypsin, pepsin and carboxypeptidase (Rugo *et al*, 1992 cited in Cantani and Micera, 2000).

The food protein substrates for proteolytic enzymes is calculated as N x f_N in food applications (Adler-Nissen, 1986b). This is the amount of nitrogen in the protein substrate multiplied by the Kjeldahl conversion factor (f_N). Casein is often used as a substrate (Mahmoud *et al.*, 1992) and Table 2.1 shows some of the many different substrates that are used in food protein hydrolysis.

Table 2.1 Substrates for food protein hydrolysis (Adapted from Adler-Nissen, 1986b)

Substrate
Casein
Whey protein concentrate
Meat
Haemoglobin
Gelatine
Fish protein concentrate
Soya proteins
Wheat gluten
Maize protein isolate

These substrates can be hydrolysed using different enzyme preparations such as pepsin, alcalase, subtilisin and trypsin. The resulting hydrolysate can differ considerably in degree of hydrolysis, molecular weight and length of the peptides depending on the enzyme used. The factors used to select the enzyme for the desired outcome include the pH optimum, thermostability, price and availability of the enzyme (Kilara, 1985).

Endopeptidases hydrolyse the peptide bonds and produce relatively large peptides. Occasionally, endopeptidases are combined with exopeptidases, which systematically remove amino acids from the N- or C terminus to

achieve a more thorough degradation of the protein (Figure 2.6) (Adler-Nissen, 1986c; Clemente, 2000).

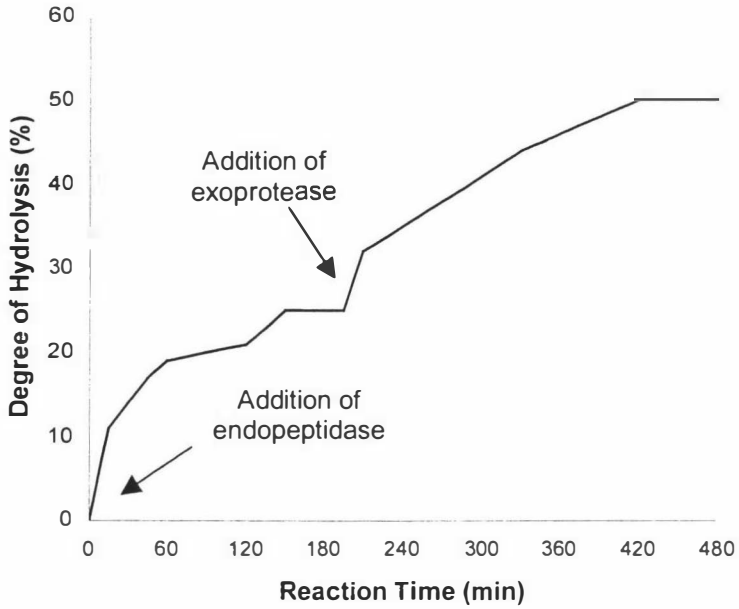

Figure 2.6 Hydrolysis curve of chickpea protein isolate obtained by sequential treatment of endopeptidase and exopeptidase enzymes (from Clemente *et al.*, 1999)

In food protein hydrolysis there are four classes of endopeptidases: aspartic-, cysteine-, metallo-, and serine proteases. Their maximum catalytic activity is found at different pH levels ranging from acid to neutral to alkaline. Unfortunately there is variation between batches of commercially available enzymes, which produces variability in the end product (e.g. protein hydrolysate). These differences are not very well documented because it is difficult to understand the factors that control this variation (Kilara, 1985).

2.8 Physical characteristics of protein hydrolysates

Proteins are usually described in part by their molecular weight (MW). For example, cows' milk proteins are in the MW range of 15,000-24,000 Dalton (Da) and soybean proteins are in the MW range of 180,000-350,000 Da. Protein hydrolysates can be extensively or partially hydrolysed. The MW of

extensively hydrolysed proteins is usually <1,500 Da and that of the partially hydrolysed proteins is >15,000 Da (Businco *et al.*, 1999). Partially hydrolysed formulae have considerably greater amounts of intact proteins than extensively hydrolysed formulae (Businco, 1994 cited in Businco *et al.*, 1999).

Table 2.2 Physicochemical and functional properties of protein hydrolysates in nutritional products (Mahmoud, 1994)

Chemical and Physicochemical Properties	**Functional properties**
Molecular size	Immunogenicity (allergenicity) Solubility Osmolality Viscosity Gelation Emulsification Clarity (turbidity) Flavour
Surface activity and hydrophobicity	Emulsification Foaming
Carbohydrate interaction	Maillard browning Colour formation Gelation Flavour formation
Mineral interaction	Solubility Thermal stability

Two of the most important properties of hydrolysed proteins are their high solubility (even during heat treatment) and their emulsifying ability. Studies have shown that even partial hydrolysis produces substantially increased solubility of the resulting hydrolysates (Mahmoud, 1994). The better solubility of the hydrolysates is due to their smaller size and the newly exposed amino

groups that increase the hydrolysates' hydrophilicity. The emulsifying properties of hydrolysed proteins are improved by controlling the extent of hydrolysis to preserve a minimum peptide length (Van Der Ven *et al.*, 2001). Too much hydrolysis, such as occurs during the manufacture of hypoallergenic protein hydrolysates, results in loss of the protein's emulsifying properties. Other properties of food protein hydrolysates are shown in Table 2.2.

2.9 Digestibility

The gastrointestinal absorption of hydrolysates, especially di- and tripeptides, is thought to be more effective than that of intact protein (Ziegler *et al.*, 1990). This is particularly helpful when intestinal absorption is reduced and/or digestive function is impaired. This is especially true at higher concentrations (Hegarty *et al.*, 1982). Grimble *et al.* (1986) showed that absorption of total nitrogen and amino acids occurred to a significantly greater extent from a low MW hydrolysate than from a hydrolysate with higher MW. Small peptides also are more rapidly absorbed than their constituent free amino acids (Matthews and Adibi, 1976; Siemensma *et al.*, 1993). In contrast, Rouanet *et al.* (1990) measured the faecal apparent digestibility of healthy, 25-30 day old Sprague-Dawley rats fed a bovine plasma protein hydrolysate diet containing 75% di- and tripeptides and the original bovine plasma protein and found that there was no difference in digestibility between the diets. A difference in the absorption of hydrolysates and the absorption of the intact protein is only likely to be apparent with proteins that are difficult to digest.

Nitrogen balance for elemental (purified AA's as protein source) and non-elemental (egg albumin as protein source) diets is similar in patients with moderate bowel dysfunction and patients with severe bowel dysfunction (Fairfull-Smith *et al.*, 1980). Keohane *et al.* (1983) concluded that if maximal absorption is required, low rather than high MW peptides should be used as the oligogopeptide based nitrogen source of elemental diets. Raimundo *et al.* (1988) concluded that when maximal absorption of nitrogen from casein

hydrolysate is required, the peptide chain length needs to be smaller than four.

2.10 Immunological characteristics

It is important to first define some terms: *antigenicity* is the capacity of molecules (antigens) to stimulate an immune response; *immunogenicity* is the capacity of molecules (immunogens) to elicit production of specific antibodies and/or to initiate specific cellular immune responses; and *allergenicity* is the capacity of molecules (allergens) to induce specific allergic responses (Anderson, 1998). Other authors can define terms differently. For example N. Cave (Massey University, pers. comm.) defines immunogenicity as the ability of dietary antigens to elicit antigen-specific immune responses in *naïve* recipients. Antigenicity essentially means the same, but could also elicit a response in an already sensitised patient, and is thus reserved for references to sensitised patients. Allergenicity describes the capacity to generate specific IgE (N. Cave, Massey University, pers. comm.). In the context of this thesis the definitions of Anderson are used.

The allergenicity of hydrolysate diets can be assessed *in vitro* or *in vivo* with the help of several immunological tests. The *in vitro* tests used are RAST (radioallergosorbent test), RAST-inhibition test, Western blot, lymphocyte proliferation assay, immunoelectrophoresis, enzyme-linked immunosorbent assay (ELISA) and ELISA-inhibition assay. *In vivo* tests used include skin and oral challenge tests. In research, the most frequently used *in vivo* technique to evaluate the immunogenic or allergenic character of protein hydrolysates is the oral sensitisation of guinea pigs (Pahud *et al.*, 1985 cited in Cordle *et al.*, 1991). Clinically, the most meaningful evaluation of the allergenicity of a food ingredient is by double-blind placebo-controlled oral challenge (Daniels, 1999).

The allergenic properties of a small number of protein hydrolysates have been evaluated in clinical studies. A study by Chirico *et al.* (1997) showed

that a partially hydrolysed formula was less immunogenic and antigenic than a non-hydrolysed traditional formula but was as immunogenic and antigenic as breast milk. Halken *et al.* (2000) found that a partially hydrolysed formula was more antigenic than an extensively hydrolysed formula. Nakamura *et al.* (1992) concluded that the antigenicity of whey protein concentrate hydrolysates prepared with trypsin and treated with an ultra-filtration membrane dropped from 1/30 to 1/200 compared with the untreated whey protein concentrate hydrolysate. However, antigenicity does not always correlate directly with MW. Nakamura *et al.* (1993) investigated the antigenicity of peptides prepared enzymatically with three different proteases. The MWs of the peptides were similar, but they showed different antigenicities. It was suggested that the presence of differing high molecular fractions might have caused the variation in the antigenicity.

2.11 Hypoallergenic diets

Partially hydrolysed diets are often called 'hypoallergenic' diets. However, this term has recently been defined by the Subcommittee on Nutrition and Allergic Disease of the American Academy of Paediatrics as a formula of which the base protein has been modified to reduce antigenicity so that 90% of subjects allergic to the base protein may tolerate the formula without symptoms (Businco *et al.*, 1999). This definition correctly implies that before a protein hydrolysate can be termed 'hypoallergenic' it must be demonstrated to actually be hypoallergenic in the patient group at risk of the allergic reaction.

Extensively hydrolysed formulae were developed for the purpose of reducing the antigenicity of the food. Extensive hydrolysis is essential to render the proteins immunologically unreactive for feeding as hypoallergenic infant formulae to allergy-prone infants (Cordle *et al.*, 1991). The functional properties of such extensively hydrolysed food proteins have not been reported.

The earliest marketed hypoallergenic formulae were made up of more than 70% free amino acids and of peptides up to 5-8 amino acids long. After these initial formulae, the hydrolysates made had a lower concentration (40-60%) free amino acids and peptides up to 10-12 amino acids long. More recent hypoallergenic infant formulae have less than 20% free amino acids and have peptides up to 10-15 amino acids long (Siemensma *et al.*, 1993). Currently, partial or extensive *in vitro* enzymatic hydrolysis of cows' milk proteins, followed by heat treatment and ultra filtration through filters with a MW cut-off value between 5,000 Da and 10,000 Da, are used to reduce cows' milk antigenicity for paediatric hypoallergenic diets (Van Beresteijn, 1995).

The first study of protein hydrolysates for use in dogs and cats was performed by Cave in 2001. Cave (2001) investigated a fish hydrolysate and a chicken hydrolysate and concluded that the chicken hydrolysate was more hydrolysed. Ninety seven percent and ninety three percent of the amino acids or peptides in the chicken hydrolysate were determined to be less than 10,000 Da and 5,000 Da, respectively (Cave, 2001). This chicken hydrolysate was used to formulate the Canine z/d® ULTRA Allergen Free diet[a] and is one of the leading partially hydrolysed formulae that are commercially available currently for veterinary use.

The ideal MW for a hydrolysed protein that will render the protein hypoallergenic is not yet known for the cat or dog (McNeill *et al.*, 2001) but the lower the MW of the protein, the lower the antigenicity. It has been suggested that peptides with MWs <1800 Da are not allergenic in humans, but it is still not clear what the optimal degree of hydrolysis is and, at present, there is no definite MW below which peptides are considered non-allergenic or non-immunogenic (Businco *et al.*, 1999).

[a] Hill's Pet Products, Topeka Kansas.

2.11 Formulation

Infant formulae are stable mixtures of emulsified fats, proteins, carbohydrates, vitamins, and minerals (Anderson *et al.*, 1982). In general, as a complete substitute for human milk, formula should provide protein at 7-16% of calories, fat at 30-54% of calories, linoleic acid at 2-3% of calories, and the remaining calories from carbohydrate sources (National Research Council, 1989). The minimum and maximum nutrient levels of infant formulae are displayed in Appendix 1.
Hydrolysed infant formulae are usually formulated with vegetable lipids and most are lactose-free and contain a small amount of carnitine (Cantani and Micera, 2000), which is thought to have a beneficial effect on fat absorption. Fat is preferably provided in the form of a mixture of medium and long chain triglycerides (Milla, 1991).

To provide a balanced amino acid content in a given protein hydrolysate certain amino acids can be supplemented (Lee, 1992). Hydrolysate manufacturers normally supply hydrolysed caseins, whey, soy, or blends without supplementation (Lahl and Braun, 1994) but specially designed vitamin and mineral supplements are available for adding to the protein hydrolysate diet. The manufacturers of the infant formula can then make final adjustments to the dietary composition by, for example, adding iron and calcium supplements.

Lactose intolerance is the most common form of carbohydrate intolerance. Hypoallergenic formulae therefore can cause diarrhoea if they include lactose. Therefore it is desirable to use other disaccharides such as sucrose or monosaccharides such as glucose in the formulation of hydrolysate formulae (Milla, 1991) or to use starch as the carbohydrate source.

Similarly, the manufacturers of protein hydrolysate diets for dogs and cats must formulate their products to meet the nutritional needs of dogs and cats. In the first instance it needs to be ensured the product meets the recommended daily nutrient intakes for either dogs or cats. This is followed

by feeding trials to ensure the product is complete and balanced and also palatable. During the formulation of the diet the manufacturer must attempt to avoid the introduction of allergenic proteins to the diet via the carbohydrate and fat sources used in the diet. Contamination of fat sources by traces of protein has previously been recognized as a problem in the human literature (Moneret-Vautrin *et al.*, 1991; De Montis *et al.*, 1993). These authors express their concern about allergenic peanut oil in milk formulas and vitamin D preparations.

2.13 Nutritional adequacy

The nutritional value of protein hydrolysates has always been an important consideration during their development because the principal use of hydrolysate formulae was for pre-term infants. Inadequacies of certain nutrients such as protein and amino acids are more likely to become apparent during infancy.

As previously mentioned, for the hydrolysate to be of most nutritional value it should be rich in low MW peptides with only low amounts of free amino acids (Silvestre, 1997). Preparations rich in small peptides from partially hydrolysed proteins have been shown to be utilised more efficiently and have a higher nutritive value than an equivalent mixture of free amino acids (Monchi and Rerat, 1993; Morato *et al*, 2000; Silk *et al*, 1982). Studies of animals and humans have shown that oligopeptides are absorbed faster and more evenly than equivalent mixtures of free amino acids (Adler-Nissen, 1986c). Rouanet *et al.* (1990) found that a hydrolysate diet containing di- and tripeptides is efficiently, but not better utilized in healthy growing rats compared to utilisation of the diet containing the original protein. Szajewska *et al.* (2001) did not find any disadvantage of protein hydrolysate formulae compared to standard protein formulae and suggested that extensive and partial protein hydrolysate formulae are at least nutritionally equivalent to standard protein formulae. Boza *et al.* (1995) also proved that enzymic hydrolysates from milk proteins have equivalent effects to their counterpart whole proteins in

supporting recovery after starvation in rats at weaning. Ribeiro *et al.* (1997) presents sound evidence that the use of diets containing fully hydrolysed protein may assist in the recovery of malnourished patients. In contrast, a study by Zarrabian *et al.* (1999) found that rats with an artificially inflamed bowel lost weight when fed a protein hydrolysate diet compared to those fed a whole protein diet. The reasons for the contradiction between the results of the study by Zarrabian et al. (1999) and the results of the other investigators quoted above are unknown. However, it is notable that the protein used in the study by Zarrabian et al. (1999) was of low digestibility. The weight loss was also associated with the nitrogen balance. The nitrogen balance in the protein hydrolysate group was worse than the nitrogen balance in the whole protein group. This was due to a greater ureagenesis in the protein hydrolysate group. The mechanisms underlying the shift towards conversion of ingested nitrogen into urea rather than into body protein are unknown, but may include differences in the absorption amino acids. Care should be taken when cows' milk protein hydrolysed formulae are given for prolonged periods to infants since no data are available on the nutritional assessment of infants fed exclusively hydrolysed formulae for several months (Cantani and Micera, 2000; Mihatsch *et al.* 2002).

After comparing three experimental protein hydrolysate preterm formulae (one based on 100% whey hydrolysate protein, one based on a mixture of 78% whey and 22% casein hydrolysed protein, and one based on a similar mixture enriched with histidine) to a standard preterm formula (based on 60% whey and 40% casein hydrolysate protein), Rigo and Senterre (1994) found that compared with the standard preterm formula, the use of protein hydrolysate formulas led to a decrease in nitrogen and phosphorus absorption without modification of retention. Compared with the standard preterm formula, all three protein hydrolysate formulas led to a significant increase in plasma threonine, and a decrease in tyrosine and phenylalanine concentrations. In addition, there was an important reduction in plasma histidine concentrations with the 100% whey hydrolysate protein diet. This was partly corrected with the diet based on a mixture of 78% whey and 22% casein hydrolysed protein. Similar values as in the control group were only

obtained with the mixture that was enriched with histidine. However, with this latter formula, low plasma concentrations of tryptophane and cystine were observed. Accordingly, the authors recommended that the nutritional adequacy of protein hydrolysate formulae for preterm infants needs to be investigated before they can be proposed for routine use. Another study by Rigo *et al.* (1994) showed that impaired growth and various biochemical abnormalities occurred in infants fed on protein-hydrolysed formulae (three different whey hydrolysate formulae, a soya-collagen hydrolysate formula, or a whey-casein hydrolysate formula) compared with human milk. As a result, the authors concluded these formulae could not be considered equivalent in terms of nutritional adequacy. Rigo *et al.* (1995) concluded there is evidence that protein hydrolysed formulae are not equivalent to whole protein formulae in terms of nutritional efficiency for preterm and term infants. Potential causes for the reported nutritional inadequacies of hydrolysate formulae are discussed in section 2.14.

In contrast, Verwimp *et al.* (1995) concluded that whey-protein hydrolysate based formulae result in normal growth and reduced allergic symptoms in infants with cows' milk protein intolerance. Following an extensive review of the literature, Blecker (1997) concluded that formulae for food-allergic infants should contain peptides of the lowest possible MW to reduce the symptoms of food allergy but the peptides should be hydrolysed to the least possible extent to provide the best nutritional outcomes.

There are only a small number of pet food companies that produce hydrolysed protein diets. These hydrolysed protein diets have passed AAFCO[b] feeding trials for the maintenance feeding of adult dogs showing them to be complete and balanced for this life stage. To the author's knowledge, however, there are no published trials evaluating the nutritional value of hydrolysed proteins in growing animals. Until such data are available, veterinary hydrolysate diets should be used for short periods only in growing animals.

[b] Association of American Feed Control Officials.

2.14 Why protein hydrolysates may be less nutritionally valuable

Rege *et al.* (1986) concluded that the low nutritive value of a pre-digested milk protein hydrolysate was most likely the result of drying the hydrolysed protein in the presence of other ingredients such as sugar. The cross linking of sugars and amino acids during the thermal drying process is thought to adversely affect the availability of certain amino acids (Bjarnason and Carpenter, 1970). The most well known of these reactions is the Maillard reaction.

Maillard reactions occur between reducing carbohydrates (e.g. lactose) and the free amino group of amino acids and yield enzyme resistant linkages that make a portion of the amino acids, particularly lysine, nutritionally unavailable. The Maillard reaction explains the fall in nutritive value of the proteins in foods containing reducing sugars that can occur even during relatively mild conditions in processing or storage (Bjarnason and Carpenter, 1969). Low availability of lysine was also found in infant formulae by Ferrer *et al.* (2000) who suggested that the thermal treatment of the formulae reduces their available lysine contents.

Enzymatic and non-enzymatic "browning reactions" of amino acids and proteins can also occur with oxidised lipids and oxidised phenols. These reactions occur during processing and storage and result in changes in the structures of the proteins, which affect their nutritional quality (Swaisgood and Catignani, 1985). The loss in nutritional quality (and potentially in safety) has been attributed to destruction of essential amino acids, decrease in digestibility, inhibition of proteolytic and glycolytic enzymes, and formation of antinutritional and toxic compounds (Friedman, 1996).

Picaud *et al.* (2001) concludes that the nitrogen absorption rate of protein hydrolysates is lower than that of intact proteins and suggests, therefore, a 10% increase in nitrogen content of protein hydrolysates compared to intact proteins. Hegarty *et al.* (1981) indicate that differences between amino acid uptake from protein hydrolysates and equivalent free amino acid mixtures are

dependent upon the concentration of the hydrolysate. There is significantly more absorption from highly concentrated hydrolysates than from hydrolysates of a lower amino acid concentration.

Another potential reason for the lower nutritive value can be the molecular form of dietary nitrogen (e.g. small peptide hydrolysates, whole proteins or free amino acids). The molecular form of nitrogen source affects body weight, nitrogen retention and small intestinal mucosa morphology (Zarrabian *et al.*, 1999). The reason for this is unknown, but may relate to differences in the absorption of amino acids. The absorption kinetics of amino acids results in differences in the distribution of free amino acids in the tissues after infusion of small peptides or free amino acids (Rérat, 1995). The imbalances that result from these differences lead to a lower metabolic amino acid utilisation, which leads to a lower nutritive value.

In contrast, Rigo *et al*. (1994) relates the impaired growth of infants fed a hydrolysed protein formula compared to human milk to the lower amount of energy available in the hydrolysed protein formula. Lastly methionine enrichment enhanced the nutritional value of soy protein hydrolysates (De La Barca *et al.*, 2000) and of buffalo and cows' milk casein hydrolysates (Hussein *et al*. 2000).

In conclusion, in most research in which proteins and protein hydrolysates are compared it has been concluded that they are not equal in nutritional value. Therefore, before protein hydrolysates are used for long-term nutritional purposes - particularly in growth - they should be carefully evaluated for their nutritional adequacy.

2.15 Bitter taste

The first hydrolysates used for clinical purposes were very unpleasant to eat and there has subsequently been a lot of research into suppressing their bitter taste. Murray and Baker (1952) discovered that the type of proteolytic enzyme used for hydrolysis markedly affects the taste of the resulting product. The bitterness is caused by peptides that have extensive hydrophobic groups at the C-terminal end (Matoba and Hata, 1972 cited in Adler-Nissen, 1986c; Stevenson *et al.*, 1998). These bitter peptides are not a major part of the hydrolysates; they account for only 5-10% of their weight. Kirimura *et al.* (1969) found that the bitter hydrophobic peptides contained neutral amino acids with large alkyl or aromatic side chains.

As a protein is hydrolysed there is reduction of antigenicity. Unfortunately, this is associated with a reduction in palatability (Cantani and Micera, 2000). The correlation between the hydrophobicity of the peptides and their bitterness can be calculated with the following formula, which is called the Q-rule: $Q - \sum \Delta f/n$ (Ney, 1971; Umetsu *et al*, 1985). This is the average free energy for the transfer of the amino acid side chains from ethanol to water, divided by the number of amino acid residues. The majority of the bitter peptides have Q-values above + 1400 cal/mole and the majority of the non-bitter peptides have Q-values below + 1300 cal/mole. This principle is valid for MWs below approximately 6,000 Da; above that MW, peptides with Q-values higher than 1,400 kcal/mole are not bitter (Pedersen, 1994). Adler-Nissen (1986c) however, showed that the Q-rule could not be directly extrapolated to mixtures of peptides in protein hydrolysates. Bitterness of the hydrolysate is not determined by the mean Q-value of all the peptides in the hydrolysate mixture. Rather it is caused by the strongly hydrophobic peptides with a high Q-value in the hydrolysate. The presence and concentration of such strongly hydrophobic peptides cannot be predicted from the mean Q value of the hydrolysate.

The bitterness of protein hydrolysates can be removed by various methods (Saha and Hayashi, 2001). These include the plastein reaction, masking or

extraction of bitter peptides. In the plastein reaction, the protein is first enzymatically hydrolysed to a low MW mixture of peptides and then enzymatically resynthesised into products which consist of insoluble, highly aggregated material having different structural, compositional and functional properties from that of the initial protein. After hydrolysis, the impurities, which cause undesirable odours, colours and flavours, are removed by treating the mixture with a protease (Dave *et al.*, 1991). Bitter peptides can also be removed by organic solvents, hydrophobic interaction chromatography, treatment with activated carbon, selective separation, treatment with enzymes such as aminopeptidase (Saha and Hayashi, 2001) or addition of gelatin (Stanley, 1981). The disadvantages of these debittering methods are that there is less hydrolysate yield and a decreased nutritional quality of the hydrolysate.

Meat hydrolysates are usually regarded as non-bitter, but a trained taste panel described a pancreatic hydrolysate of chicken meat as bitter (Stanley, 1981). Free glycine reduces the bitter taste of chicken meat hydrolysate when added during the hydrolysis process. Nishiwaki *et al.*, (2002) reported a method using an aminopeptidase from the edible basidiomycete *Grifola frondosa* to debitter the enzymatic hydrolysates of soy protein and milk casein. Another potential method to improve the bitter taste of protein hydrolysates was found by Stevenson *et al.* (1998) who described the protease-catalysed condensation of peptides. Capiralla *et al.* (2002) described an endopeptidase from *Halobacterium halobium* S9, which can be used for debittering of meso, cheese and protein hydrolysates in the food processing industry.

Some caution should be expressed on the experience of bitterness, as a negative experience of bitterness is not homogenous between species. Both cats and dogs respond well to hydrolysates, which are widely used as enhancers of palatability in dry foods (N. Cave, Massey University, pers. comm.).

2.16 Other adverse effects

Loose stools or diarrhoea are a well-known side effect of hypoallergenic hydrolysed protein formulae in infant feeding (Mihatsch *et al.*, 2002). The diarrhoea is thought to be due to an excessive osmotic load (see Chapter 4). Severe diarrhoea and even death has been reported in piglets fed a peptic hydrolysate of soya protein or a tryptic digest of skim milk (Cunningham and Brisson, 1957; Pettigrew *et al.*, 1977). The reason for this is not clear, but the authors suggest it might have happened because piglets at this young age (weaned at 1 to 2 days of age) may require certain physiologically active components of milk for normal gastrointestinal function. The activity of these components in the milk was thought to be destroyed by trypsin.

Hypersensitivity (IgE-mediated allergic reaction) to commercially available hypoallergenic infant formulae based on cows' milk protein hydrolysates has been reported (Heyman *et al.*, 1990; Bock, 1990; Lifschitz *et al.*, 1988). Van Beresteijn (1995) concludes that IgE-mediated allergic reactions caused by the consumption of hypoallergenic infant formulae based on cows' milk proteins cannot be attributed to one specific protein. There are differences in the individual patterns of sensitisation to various cows' milk proteins. Cantani and Micera (2000) reviewed the literature on studies done with hydrolysed protein formulae and report a range of different allergic reactions.

There are some reports of specific deficiencies or diseases occurring in infants fed formulae due to technical errors in the manufacturing of the formulae (Anderson *et al.*, 1982). Table 2.3 shows the symptoms of reactions to highly hydrolysed casein formulae collated from different studies and some examples of deficiencies and diseases that occurred due to manufacturing errors.

Table 2.3 Symptoms of reactions to highly hydrolysed casein formulae (Adapted from Cantani and Micera, 2000 and Anderson *et al.*, 1982)

Symptoms	
Anaphylaxis	Obstructive symptoms
Atopic dermatitis worsening	Pallor
Bloating of the face	Proctorrhagia
Bloody stools	Repeated haematochezia
Bronchospasm	Rhinitis
Classical IgE-reactions	Scurvy
Clinical intolerance	Skin lesions
Cows' milk enterocolitis	Somnolence
Diarrhoea	Stridor
Enterocolitis with haematochezia	Systemic/generalised urticaria
Epidermal and retinal bleeding	Vitamin A, C, D deficiencies
Failure to thrive	Vomiting
Haematemesis	Worsening itch
Labial angioedema	

Another concern is microbial contamination during the manufacture. This may alter the nutritional quality of the protein hydrolysate as a result of microbial growth and metabolism of essential amino acids (Anderson *et al.*, 1982). Microbial contamination can cause diarrhoea and subsequent fluid and electrolyte imbalances. To prevent this problem the quality control on microbiological safety has to be very good.

2.17 Commercially available hydrolysate diets for dogs and cats

There are now several protein hydrolysate diets available on the market for use in dogs and cats. These include Hill's Canine z/d® ULTRA Allergen Free, Purina Veterinary Diet HypoAllergenic (HA) and Royal Canin Hypoallergenic diet.

The use of proteolytic enzymes to remove allergenicity has been successfully used in the production of hypoallergenic infant formulae for infants with cows' milk allergy, but this approach has been rarely employed with other allergic

food (Hefle, 1999 cited in McNeill *et al.*, 2001). Although theoretically superior, there is as yet little evidence that diets containing protein hydrolysates are clinically superior to existing selected protein (exclusion) diets for either the diagnosis or management of dietary sensitivities in dogs and cats (McNeill *et al.*, 2001). There have been few *in vivo* clinical trials to evaluate the effectiveness of hydrolysed protein diets in animals with allergic responses to various proteins in their diets (Hefle, 1999 and Gortler *et al.*, 1995 cited in McNeill *et al.*, 2001).

One such *in vivo* clinical trial has been performed by Marks *et al.* (2002) who concluded that the feeding of HA HypoAllergenic™ brand Canine Formula[c] to dogs with Inflammatory Bowel Disease was associated with partial to complete resolution of clinical signs. In this study there was a dramatic clinical improvement in some dogs that previously lacked response to selected protein hypoallergenic diets and medical therapy. This trial suggests that diets containing hydrolysed protein may prove to be an aid in the management of dietary sensitivities in dogs, but more research on this topic is required. A second *in vivo* trial by Dossin *et al.* (2002) on soy isolate hydrolysate in the management of inflammatory bowel disease (IBD) in dogs showed similar results. This preliminary study suggested that a soy isolate hydrolysate diet could be useful in the clinical management of IBD, even in severe cases, and might be a good alternative to the use of corticosteroids. Jackson *et al.* (2003) demonstrated that a soy hydrolysate diet was well tolerated by a majority of dogs with confirmed cutaneous reactivity to corn and soy and this soy hydrolysate diet may be an appropriate choice for the long-term management of individuals allergic to soy or corn protein.

[c] Nestlé Purina PetCare Company, St. Louis, MO.

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Chapter 3

Use of a rat model to assess the apparent ileal digestibility of Canine z/d® ULTRA Allergen Free

3.1 Introduction

There have been anecdotal reports from pet owners and veterinarians in private practice that dogs can develop diarrhoea when fed the original formulation of the Canine z/d® ULTRA Allergen Free[a] diet[b].

Diarrhoea can result from a number of different causes of which many are unknown (Guilford, 1996a). Acute diarrhoea is most often due to irritation of the gut mucosa by infections, toxins (Berry and Levett, 1986) or parasites. Acute diarrhoea can also result from sudden changes in diet. Diet changes can lead to diarrhoea because of saturation of digestive and absorptive mechanisms for the novel nutrient. This is most commonly encountered when changing from a complete, dry, cereal-based diet to canned or fresh meat (Murdoch, 1986) or from a low fat to a high fat diet or a low carbohydrate to a high carbohydrate diet (G. Guilford, Massey University, pers. comm.). Different foods require a different range of enzymes for digestion (Agar, 2001a) and/or an increase in the amount of pancreatic and intestinal digestive enzymes secreted (Guilford and Strombeck, 1996). The body needs time to produce these enzymes to allow adaptation to the new food. The food particles that don't get digested draw fluid into the gut through osmosis resulting in osmotic diarrhoea.

[a] Hill's Pet Products, Topeka Kansas.
[b] Personal communication of P. Roudebush, Hill's Pet Products, Topeka, Kansas, 1999.

3.2 Poor digestibility of a diet as a cause of diarrhoea

Minimum dry matter digestibility for a good quality dry food is considered to be near 75% (Lewis and Morris, 1984 cited in Huber *et al.*, 1986). The digestibility of most cereal and soy products is in the range of 71-80%, whereas meats are digested at levels of 80-90% (Kendall and Holme, 1982; Meyer, 1984; Brown, 1989). Digestibilities as high as 89%, 95%, and 88% for crude protein, crude fat, and carbohydrate, respectively, can occur in dry-type premium pet food (Case, 1999; Case *et al.*, 2000a). According to Zentek and Meyer (1995), nutrient absorption seems to be influenced by breed type. The authors report dry matter digestibilities for dry diets from 78.3% to 80.3% for great danes and 79.8% to 88.5% for beagles. The National Research Council (National Research Council, 1985) suggests a digestibility coefficient of 85% be used for carbohydrate in commercial dog foods and a 90% digestibility coefficient for fat.

The digestibility of amino acids in feeds is highly variable. For example amino acids in casein are almost completely absorbed during digestion whereas more than half of the amino acids in meat and bone meal can remain unabsorbed in the digestive tract (Moughan and Donkoh, 1991). It is uncertain whether poor digestibility of amino acid leads to diarrhoea.

Fat has a number of different roles in dog food. It contributes to the palatability and texture of the food; it provides energy in a very concentrated form; it has numerous metabolic and structural functions; and it provides a source of essential fatty acids (EFAs) (Agar, 2001b). The NRC recommends a 90% digestibility coefficient be used for fat in commercial dog foods (National Research Council, 1985). Maldigestion and malabsorption of fat and subsequently bacterial metabolism of fatty acids and bile salts in the colon can result in secretory diarrhoea (Batt and Burrows, 1994; Case *et al.*, 2000b) in addition to the osmotic diarrhoea due to the malabsorption of nutrients. As a result, restriction of dietary fat intake when the diarrhoea is of small intestinal origin will reduce diarrhoea (Watson and Markwell, 1997).

The process of assimilation of fat is fairly complex and therefore quite easy to disrupt.

Similarly, poorly digested carbohydrates can, among other problems, cause osmotic diarrhoea. Lactose (a disaccharide) is often included in dog diets, but because lactose is not well tolerated by most dogs it can result in watery diarrhoea (Burger, 1982; Guilford, 1996[b]). Several raw starches are poorly digested, but cooked starch and finely ground starch is efficiently digested and absorbed by dogs (Romsos *et al*., 1978; Bisset *et al*., 1997).

The inclusion of optimal amounts of fibre in the diet is necessary for the normal functioning of the gastrointestinal tract. Increasing amounts of fibre (a carbohydrate) in the diet can reduce the digestibility of the diet and increases faecal bulk. For example Mitaru and Blair (1984) concluded that the protein digestibility values for their control and Alphafloc diets were higher ($p<0.05$) than for their (high fibre) canola and soya bean hull diets when fed to rats. Lignin is reported to have a detrimental effect on protein digestibility, but cellulose has no adverse effect on protein digestibility (Mitaru and Blair, 1984; Shah *et al*. 1982). In general, dogs digest foods of animal origin better than those of plant origin. This difference is primarily the result of the presence of lignin, cellulose, and other components of fibre in plant ingredients (Case *et al*., 2000[a]). Mitaru and Blair (1984) also concluded that the dry matter digestibility of a (low fibre) control diet was higher than high fibre-containing diets.

3.3 Measurement of digestibility of a diet

Traditionally, the digestibility of a diet has been determined *in vivo* by calculating the difference between the nutrient intake in the food and the nutrient output in faeces, on a dry matter basis. Digestibility is usually expressed as a percentage, known as the digestibility coefficient (DC).

$$\text{Digestibility coefficient} = \frac{\text{Intake} - \text{faecal output}}{\text{Intake}} \times 100$$

This can be determined either directly, by measuring the total amounts of nutrients ingested and excreted, or indirectly, by measuring the ratio of the nutrients to an indigestible marker such as titanium (see section 3.6.2). The usual method of measuring digestibility is feeding the diet to a number of animals after which the faeces are collected throughout a predesignated time period. It is also possible to determine the digestibility of a diet *in vitro* by reproducing the digestive reactions which take place in the alimentary tract of the animal (McDonald *et al.*, 1995).

The usual *in vivo* method of calculating digestibility through measuring nutrient output in the faeces has been criticised because of the inaccuracies resulting from microbial action in the large intestine (Zebrowska, 1975, cited in Moughan *et al.*, 1984; McNeil, 1988). The microorganisms in the large intestine may metabolise dietary and endogenous proteins and amino acids. This metabolism can artificially increase the digestibility coefficient. For this reason, this method of calculating digestibility is called the 'apparent faecal digestibility'. To overcome this problem, the apparent ileal digestibility measurement method has been developed. In this method the digesta (with or without an indigestible marker) is collected at the terminal ileum of the animal to avoid metabolism of nutrients in the large intestine (van Weerden, 1989). The impact of microbial degradation in the large intestine on estimation of protein digestibility coefficients has been observed in several monogastric species with notable differences in faecal and apparent ileal

digestibility coefficients (Moughan and Donkoh, 1991). It is now generally accepted that measuring digestibilities at the terminal ileum gives a more reliable estimate of the digestibility of nutrients (Rerat, 1981; Moughan and Donkoh, 1991; Sauer and Ozimek, 1986; van Weerden, 1989;).

To determine *true* digestibility the endogenous losses should be taken into account. To do this a correction is made for the non-dietary components, such as bacteria, hair, digestive secretions, mucus and cells, that may be present in the digesta (Moughan and Donkoh, 1991; Rutherfurd and Moughan, 1998). True digestibility is a fundamental property of the food. It is not affected by dietary features such as the protein content of the diet under which the food is fed to the animal (Darragh and Hodgkinson, 2000). True digestibility is usually calculated when it is necessary to get a better description of the uptake of amino acids from the digestive tract. It was considered that there was no requirement to determine true digestibility in this study as the study's aim (see paragraph 3.5) could be met by the measurement of apparent digestibility.

The measurement of digestibilities at the terminal ileum requires invasive procedures or euthanasia of the animals used in the digestibility study. This is impractical in companion animals for ethical reasons. Therefore, a previously validated rat model for assessing the digestibility of diets by dogs was used (Sritharan, 1998). In this model, the apparent ileal digestibility of amino acids, nitrogen, crude protein, fat and carbohydrates in the dog can be predicted from the respective rat values (Sritharan, 1998).

3.4 Why the rat as a model animal for the dog?

In their book, *The Principles of Humane Experimental Technique*, Russell and Burch (1959) argue for replacement, reduction and refinement in the use of animals in scientific research. Replacement is the replacement of the animal experiment by a research method that will gather the same result but doesn't need live animals. Reduction of the number of animals needed in an experiment can be obtained by standardisation of the animal population and experimental procedures so the variation of the results decreases. Refinement is the striving to decrease the discomfort caused by the experimental method, e.g. the expansion of the knowledge of the biological properties of the animal and the 'translation' of this knowledge into optimal housing, nutrition and care of the animal.

Model animals are often used for ethical or practical reasons because direct measurements on the animal of interest may be difficult or even sometimes impossible to obtain (Sritharan, 1998). The rat is considered a suitable mammalian model for nutritional research. It is an animal that is easy to keep in a laboratory, is standardised, allows more control over the experimental conditions and continues to gain weight for a long period after weaning. Gaining weight for a long period after weaning is useful in nutritional studies (Dunn *et al.*, 1947). The rat is also inexpensive to obtain and keep and digesta sampling techniques have been developed for this animal. The rat has already been validated as a model animal for digestion in pigs, humans and dogs (Bodwell *et al.*, 1980; Pelletier *et al.*, 1983; Moughan *et al.*, 1984; Donkoh *et al.*, 1994; Sritharan, 1998).

3.5 Objective of the study

The aim of this study was to assess the apparent ileal digestibility of the Prescription Diet® Canine z/d® ULTRA Allergen Free using a rat model of apparent ileal digestibility to determine if poor digestibility may be contributing to the diarrhoea that has been reported with use of this diet.

3.6 Materials and methods

3.6.1 Animals, housing and diet

Eight healthy male Sprague Dawley rats with an initial body weight range of 221 to 232 g (mean ± SEM, 225.1 ± 1.46 g) were used in this study. The rats were supplied by the Small Animal Production Unit of Massey University, Palmerston North, New Zealand. The Massey University Animal Ethics Committee approved the experimental protocol. The standard diet of the rats was Diet '86, which was provided by the Massey University Feedmill, Palmerston North, New Zealand. The rats were kept individually in raised stainless steel cages with wire mesh floors at a temperature of ± 23°C. During the study the rats were fed the Prescription Diet® Canine z/d® ULTRA Allergen Free. This diet was ground to a powder.

3.6.2 Study protocol

Titanium oxide was added to the diet as an indigestible marker to be able to calculate digesta flows. This was done by weighing three grams (0.3%) of titanium oxide and mixing this thoroughly with one kilogram of the ground diet.

The rats were fed hourly for 10 minutes between 8 am and 5 pm. Fresh water was available *ad libitum*. The hourly feeding regimen was used to obtain a constant flow of digesta at the terminal ileum on the day of digesta sampling. The food intake of every rat was recorded after each meal. On the 12th day of

the experiment the rats were suffocated with carbon dioxide and decapitated. Twenty cm of the ileum immediately anterior to the ileocaecal junction was then dissected from the rest of the bowel (Figure 3.1).

Figure 3.1. Dissection of ileum

The outside of the dissected ileum was washed with distilled deionised water to remove any blood and hair and then carefully blotted using an absorbent paper towel (Figure 3.2). The ileal contents were gently flushed out with distilled deionised water into a plastic bag, using a syringe (Figure 3.3). The samples were frozen at -20° C immediately after collection and subsequently freeze-dried.

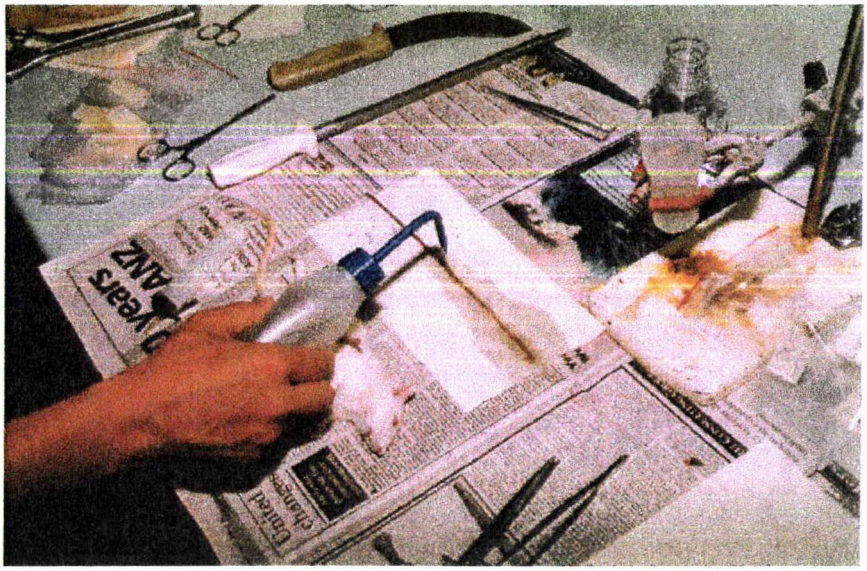

Figure 3.2. Washing of ileum

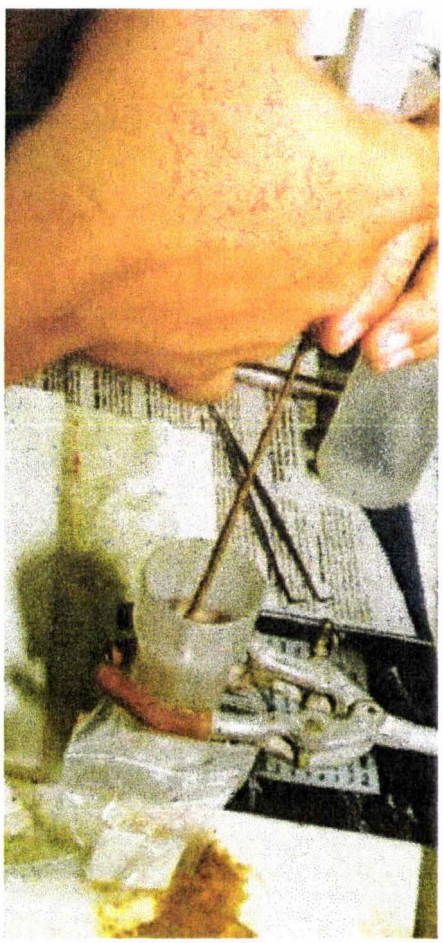

Figure 3.3. Flushing of ileal contents

3.6.3 Chemical analysis

The ileal samples and the diet were subjected to analysis of dry matter, organic matter (crude protein, fat, carbohydrates), titanium, nitrogen and amino acids. Dry matter was determined in duplicate by drying samples at 105° C for 16 hours (AOAC, 1980), while ash was determined by heating the samples at 550° C for 16 hours (AOAC, 1980). Crude protein was determined indirectly by measuring the nitrogen content and multiplying by a conversion factor of 6.25 (Rutherfurd and Moughan, 2000). This conversion factor is based on the assumption that all proteins contain approximately 16% nitrogen. Carbohydrates were calculated by difference. The titanium contents of diet and ileal samples were determined using a colorimetric assay. The samples were ignited at 500°C to burn off all organic material. The remaining minerals were digested to release titanium (Short *et al*., 1996). Total nitrogen was determined using the Kjeldahl method. Duplicate crude protein samples (± 5 mg) were hydrolysed in 1 ml of 6M glass-distilled HCL containing 0.1% phenol for 24 hours at 110 ± 2° C in glass tubes sealed under vacuum. Amino acids were determined using ion exchanged HPLC system (Waters, Millipore, Milford, MA) employing postcolumn derivatisation with ninhydrin (Hendriks *et al*., 1997). Proline was detected at 440 nm while other amino acids were detected at 570 nm. Cysteine and methionine were determined from performic acid oxidation. No correction was made for loss of amino acids during hydrolysis and the weight of amino acids were calculated using free amino acids molecular weights.

3.6.4 Data analysis

The apparent ileal digestibility of nutrients (amino acids, nitrogen, dry matter and organic matter) were calculated using the following equation (units are mg/g dry matter):

Apparent ileal nutrient digestibility =

$$\frac{\text{nutrient concentration in the diet} - \text{corrected nutrient concentration in the digesta}}{\text{nutrient concentration in the diet}}$$

Nutrient concentrations in the digesta were calculated using the following equation (units are mg/g dry matter):

Corrected nutrient concentration in the digesta =

$$\text{nutrient concentration in the digesta} \times \frac{\text{Titanium concentration in diet}}{\text{Titanium concentration in digesta}}$$

The apparent ileal nutrient digestibility of the diet in dogs can be predicted using the following regression equation:

$$Y = 0.32 + 0.65\ X$$

Where X is the apparent ileal digestibility coefficient in rats and Y the apparent ileal digestibility coefficient in dogs (Sritharan, 1998).

This regression equation originated from results of research into the comparison of the amino acid digestibility of a commercial dry dog food fed to the adult rat and dog. There was a significant linear regression between the apparent amino acid digestibility in the rat and the apparent amino acid digestibility in the dog.

3.7 Results

All the rats remained healthy throughout the experiment although two animals lost some weight over the 12 days. The rats weighed 225.1 ± 1.46 g (mean ± SEM) on the 1st, 226.5 ± 9.19 g on the 8th and 245.4 ± 11.66 g on the 12th (last) day of the experiment. The average weight gain of the rats was 20.4 ± 10.55 g (mean ± SEM) over the 12-day period and the two rats that lost weight lost on average 23.4 g ± 17.6 g (mean ± SEM).

The food intake of the rats stabilised four days after the start of the study. The average food intake was 11.6 ± 0.85 g/d (mean ± SEM) over the whole 12-day period and after stabilisation the food intake was 14.4 ± 0.92 g/d (mean ± SEM).

Table 3.1 shows the nutrient composition of the Canine z/d® ULTRA Allergen Free diet[c].

[c] Provided by nutrition laboratory of the Institute of Food, Nutrition and Human Health, Massey University.

Table 3.1 Nutrient composition of Canine z/d® ULTRA Allergen Free

Ingredient	Amount *(g/kg dry matter)*
Nitrogen	30.2
Crude protein	188.8
Fat	78
Carbohydrates	683.9
Ash	49.3
Aspartic acid	16.4
Threonine	7.9
Serine	6.2
Glutamic acid	21
Proline	8
Glycine	8.6
Alanine	10.6
Valine	10.8
Isoleucine	8.3
Leucine	15.6
Tyrosine	5.8
Phenylalanine	7.9
Histidine	4.5
Lysine	12.1
Arginine	9.8
Cysteine	2.2
Methionine	5.3

Table 3.2 shows the mean apparent ileal digestibility coefficients of amino acids, nitrogen, fat, carbohydrates, dry matter and organic matter in the rats fed the Canine z/d® ULTRA Allergen Free diet. The table also shows the predicted apparent ileal digestibility coefficients of the nutrients in dogs, calculated from the rat values using the regression equation described above.

Table 3.2 Apparent ileal digestibility coefficients in rats and dogs

Amino acid	Rat (%) measured apparent	Dog (%) calculated apparent
Aspartic acid	75.7	81.2
Threonine	76.5	81.7
Serine	72.5	79.1
Glutamic acid	82.1	85.4
Proline	78.7	83.1
Glycine	69.5	77.2
Alanine	84.6	87.0
Valine	81.9	85.2
Isoleucine	82.6	85.7
Leucine	84.9	87.2
Tyrosine	82.9	85.9
Phenylalanine	81.8	85.2
Histidine	73.8	79.9
Lysine	84.7	87.0
Arginine	87.5	88.9
Cysteine	44.0	60.6
Methionine	87.7	89.0
Crude Protein	77.5	82.4
Crude Fat	92.5	92.1
CHO	89.0	89.9
Dry matter	84.6	87.0
Organic matter	87.0	88.6

3.8 Discussion

This assessment of apparent ileal digestibility of the Canine z/d® ULTRA Allergen Free diet using the rat model demonstrated that the digestibility of crude protein, crude fat and carbohydrate is 82.4%, 92.1% and 89.9% respectively. These data compare favourably with digestibility data reported in the literature. Reported digestibility data for commercial dry dog foods range from 69.3% to 89.0% for crude protein, 69.1% to 95.0% for crude fat and 69.4% to 88.0% for carbohydrate (Huber *et al.*, 1986; Case, 1999; Kendall *et al.*, 1982). Premium pet foods usually have slightly higher digestibility coefficients than the NRC suggests (Case, 1999).

The measured and calculated apparent digestibilities of cysteine are low compared to the digestibilities of the other amino acids in the diet. This might be due to the fact that rats eat their hair, which is high in cysteine. The amount of endogenous cysteine and the amount of cysteine in the diet is low. The amount of cysteine in the digesta is high (due to hair in the digesta). This results in a disproportionately low apparent digestibility.
Due to the limited amount of digesta collected, there is a possibility of a bias in results due to unrepresentative sampling of digesta (Darragh and Hodgkinson, 2000). When the animal is fed frequently before slaughter, however, the variability of data derived is no greater than that found with cannulated animals (Donkoh *et al.*, 1994).

In summary, the present data show that the apparent ileal digestibility of the Canine z/d® ULTRA Allergen Free diet was high. It is therefore unlikely that poor digestibility contributes to the diarrhoea that has been reported in dogs fed this product.

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Chapter 4

Osmolarity of three different dog diets

4.1 Introduction

Osmosis is the movement of a solvent (such as water) through a permeable membrane from a solution that has a low concentration of solutes to a solution that has a high concentration. Osmolarity is defined as the osmotic pressure of a solution expressed in osmols or milliosmols per litre of the solution (Anderson, 1998).

In most diets, the main determinants of the osmolarity are electrolytes, soluble minerals, and simple carbohydrates (Mahmoud, 1994). Because of their large size, macronutrients such as protein, starch and triglycerides do not contribute greatly to dietary osmolarity. However, hydrolysis of protein produces a large amount of small size particles, such as amino acids and peptides, which can increase the osmolarity of the resulting product (Mahmoud, 1994; Milla, 1991; Sawatzki *et al.*, 1994; Linblad, 1978 cited in Boza *et al.*, 1995; Mihatsch *et al.*, 2001). The more hydrolysed the protein source of the product, the higher the osmolarity of the product.

A diet with a high osmolarity can result in diarrhoea because of the large amounts of water that may be drawn into the small intestine. This is called osmotic diarrhoea. The appearance of osmotic diarrhoea is characterized by large volumes of watery diarrhoea that can be resolved by fasting for 24 to 48 hours (Guilford and Strombeck, 1996). High osmolarity is also a stimulus for gastrointestinal inflammation (Madara and Trier, 1987; Kotz *et al.*, 1992 cited in Guilford, 1996[b]), which can lead to diarrhoea. Norris (1973) showed that the osmotic equilibrium following the application of a solution with a high osmolarity into the small intestine resulted in an increase in luminal contents and a subsequent decrease in tissue water fluid and venous outflow from the

segment of the ileum under study. Damage to the mucosa has been observed in animals fed very high osmolar (1,000-2,000 mosmol/kg) diets (Teichberg *et al*, 1978 cited in Billeaud *et al*., 1982). A hypertonic solution in excess of 410 mOsm/L which is in contact with the intestinal mucosa for 30 minutes or more produces mucosal damage (Kameda *et al*., 1968). The changes that occurred in the villi could be directly attributed to the dehydrating effect of the hyperosmotic solution on the epithelium.

Low osmolality of a nutrient solution decreases intraluminal water flow rates in the upper intestine without affecting the absorption rates of total nitrogen and carbohydrate. Therefore, hypo-osmolar solutions might lower the water loss in patients with extensive short bowel intestinal resection (Pfeiffer *et al*., 1998). The World Health Organization (WHO) advices Oral Rehydration Solution (ORS) as the standard treatment for dehydration caused by diarrhoea. Recently research has been directed to the osmolarity of these ORS as the standard WHO ORS has a reasonably high osmolarity of 311 mmol/l (El-Mougi *et al*., 1996). It is suggested that hypo-osmolar ORS is clinically more effective than standard ORS and may thus be advantageous for use in the treatment of children with persistent diarrhoea (El-Mougi *et al*., 1996; Dutta *et al*., 2000; Hahn *et al*., 2001; Sarker *et al*., 2001).

4.2 Objective of the study

The objective of this study was to compare the osmolarity of Hill's Prescription Diet Canine z/d® ULTRA Allergen Free to a standard maintenance diet and to a diet formulated for the treatment of diarrhoea.

4.3 Materials and methods

4.3.1 Diets

The diets chosen for this study were Prescription Diet Canine z/d® ULTRA Allergen Free[a] – a diet based on a protein hydrolysate; Prescription Diet Canine i/d®[a] – a diet formulated for the treatment of dogs with gastrointestinal conditions but which does not contain a protein hydrolysate, and Science Diet® Canine Maintenance[a] – a diet formulated for the feeding of healthy adult dogs.

The proximate analyses of these diets are compared in Table 4.1 and the ingredients of these diets are listed in Table 4.2.

Table 4.1 Proximate analysis of Canine z/d® ULTRA Allergen Free, Canine i/d and Canine Maintenance[b]

	Canine z/d ULTRA Allergen Free % Dry Matter	Canine i/d % Dry Matter	Canine Maintenance % Dry Matter
Protein	17.10	26.40	21.50
Fat	13.80	13.60	13.00
Carbohydrate (NFE)	61.40	52.30	60.86
Crude Fibre	2.90	1.20	3.00
Calcium	0.67	1.13	0.50
Phosphorus	0.51	0.83	0.40
Sodium	0.30	0.46	
Potassium	0.64	0.92	
Magnesium	0.054	0.092	
Chloride	0.92	1.10	

[a] Hill's Pet Products, Topeka, Kansas.
[b] Provided by Hill's Pet Products, Topeka, Kansas.

Table 4.2 Ingredients of Canine z/d® ULTRA Allergen Free, Canine i/d and Canine Maintenance[c]

Diet	Ingredients
Canine z/d® ULTRA Allergen Free	Starch, Hydrolysed Chicken Liver, Vegetable Oil, Powdered Cellulose, Hydrolysed Chicken, Dicalcium Phosphate, Calcium Carbonate, Glyceryl Monostearate, Potassium Chloride, Salt, Choline Chloride, DL-Methionine, Taurine, Ferrous Sulfate, Zinc Oxide, Copper Sulfate, Manganous Oxide, Calcium Iodate, Sodium Selenite, Vitamin A Supplement, D-Activated Animal Sterol, Vitamin E Supplement, Antioxidant, Niacin, Thiamine, Calcium Pantothenate, Pyridoxine Hydrochloride, Riboflavin, Folic Acid, Biotin, Vitamin B12 Supplement
Canine i/d®	Ground Corn, Brewers Rice, Dried Egg Product, Poultry By-Product Meal, Corn Gluten Meal, Animal Fat, Soy Fibre, Dicalcium Phosphate, Natural Flavour, Iodised Salt, Choline Chloride, Potassium Citrate, Potassium Chloride, Calcium Carbonate, Vegetable Oil, Taurine, Antioxidant, Ferrous Sulfate, Zinc Oxide, Copper Sulfate, Manganous Oxide, Calcium Iodate, Sodium Selenite, Vitamin A Supplement, D-Activated Animal Sterol, Vitamin E Supplement, Niacin, Thiamine, Calcium Pantothenate, Pyridoxine Hydrochloride, Riboflavin, Folic Acid, Biotin, Vitamin B12 Supplement
Canine Maintenance	Ground Corn, Poultry By-Product Meal, Soybean Meal, Animal Fat (Preserved with BHA, Propyl Gallate and Citric Acid), Natural Flavour, Vegetable Oil, Dried Egg Product, Flaxseed, Iodised Salt, Choline, Chloride, Calcium Carbonate, Ferrous Sulfate, Zinc Oxide, Copper Sulfate, Manganous Oxide, Calcium Iodate, Sodium Selenite, Vitamin A Supplement, D-Activated Animal Sterol, Vitamin E Supplement, Niacin, Thiamine, preserved with BHT and BHA, Calcium Pantothenate, Pyridoxine Hydrochloride, Riboflavin, Folic Acid, Biotin, Vitamin B12 Supplement

[c] Provided by Hill's Pet Products, Topeka, Kansas.

4.3.2 Study protocol

The diets were ground in a grinder[d] until a powdery state was reached. Three 10-gram samples of each of the ground diets were then placed in beakers and 40 ml, 80 ml or 160 ml of distilled water was added to yield three weight-by-volume concentrations of 1:4, 1:8 and 1:16. The solution was stirred for 10 minutes using a magnetic stirrer. The solution was then centrifuged[e] for 10 minutes on 2,900 rotations per minute (1,700 xg). Two ml of the solution was pipetted in to an osmometer[f]. The osmolarity was determined by the freezing-point depression method. The protocol was repeated to get a duplicate sample.

4.3.3 Data analysis

The SAS system[g] was used for the statistical analysis of the results.
The osmolarities of the Prescription Diet Canine z/d® ULTRA Allergen Free, Prescription Diet Canine i/d® and Science Diet® Canine Maintenance diets were analysed using the MIXED procedure of SAS with a linear model that included the effect of diet and concentration.
Expected osmolarity values outside of the concentrations studied for each of the diets were predicted using a linear regression model. The estimates of the intercept and the slope for each diet were used to predict the osmolarities at 1:1 and 1:2 concentrations. The linear models were:

$\hat{y} = 233.5 - 11.6\ x$ for diet Canine i/d®

$\hat{y} = 169.0 - 8.4\ x$ for diet Canine Maintenance

$\hat{y} = 392.0 - 18.9\ x$ for diet Canine z/d® ULTRA Allergen Free

where $\hat{y}$ is the predicted osmolarity at concentration x.

[d] Breville, Model CG-2.
[e] International Equipment Company, IEC Centra GP 8R, Needham Heights, USA.
[f] Advanced Instruments Inc., Model 3D2, Massachussetts.
[g] SAS Institute Inc., Release 8.02, Cary, NC, USA.

4.4 Results

Table 4.3 shows the least square means of the osmolarities of the three different diets. The osmolarity of the Canine z/d® ULTRA Allergen Free diet was significantly different from the osmolarities of the Canine i/d® and Canine Maintenance diets at respectively p=0.0463 and p=0.0165. The osmolarity of the Canine i/d® diet and the osmolarity of the Canine Maintenance diet were not significantly different from each other at p=0.3240.

Table 4.3 Least square means of the osmolarities of three dog diets

Diet	LS means
Canine z/d® ULTRA Allergen Free	211.75[a]
Canine i/d®	125.67[b]
Canine Maintenance	91.75[b]

Table 4.4 shows the least square means of the osmolarities of the three different concentrations. The osmolarity of the 1:4 concentration was significantly different from the osmolarities of the 1:8 and 1:16 concentrations at p=0.0204 and p=0.0049, respectively. The osmolarity of the 1:8 concentration was not significantly different from the osmolarity of the 1:16 concentration at p=0.1307.

Table 4.4 Least square means of the osmolarities of three weight/volume concentrations

Concentration	LS means
1:4	237.08[a]
1:8	124.67[b]
1:16	67.42[b]

The mean osmolarity in milliosmoles (mOsm) of the three different dog diets are displayed in Figure 4.1.

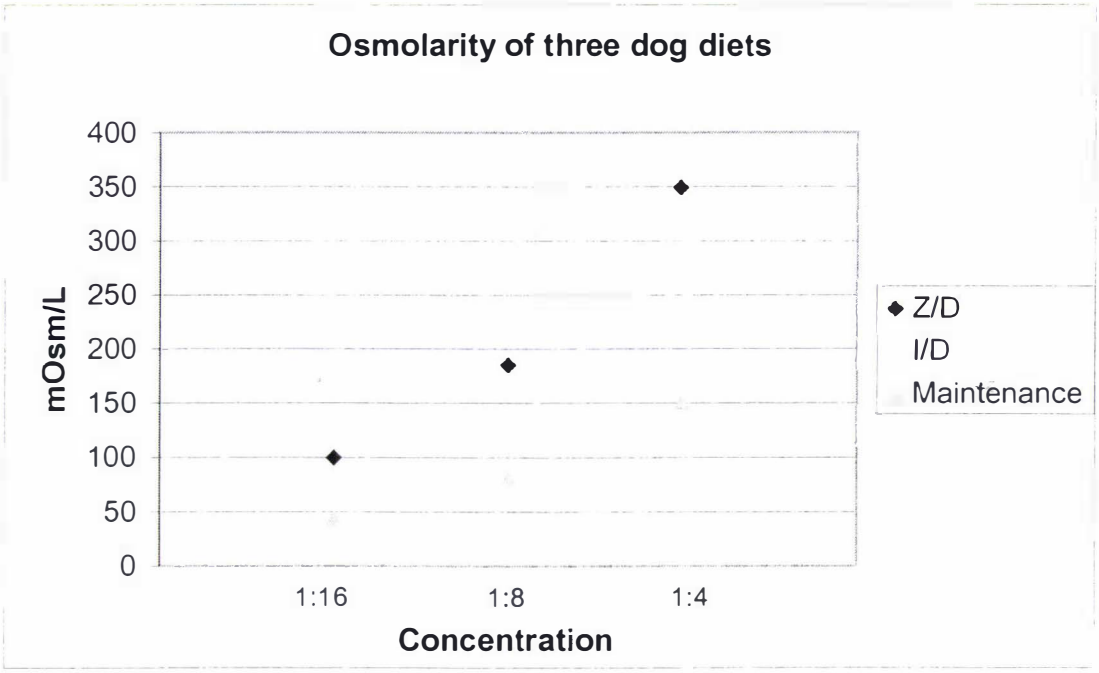

Figure 4.1 Mean osmolarity of three dog diets

Extrapolation of these data is shown in Figure 4.2. The data points shown in Figure 4.2 are exactly the same as those shown in Figure 4.1. The line shown is the extrapolated line derived from those data points.

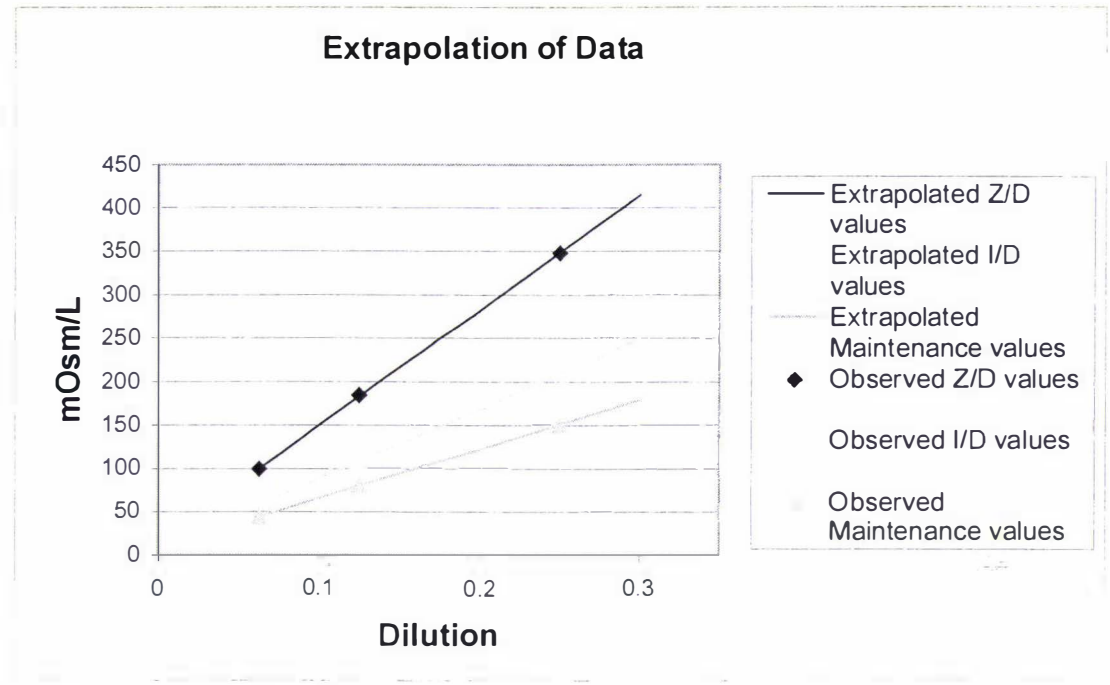

Figure 4.2 Extrapolation of osmolarity data

4.5 Discussion

No previously published techniques on measuring dietary osmolarity were found in the literature. Therefore the study relied on a *de novo* protocol.

At every weight/volume concentration of the diet solution measured, the osmolarity of Canine z/d® ULTRA Allergen Free diet was approximately 2-fold higher than the other two diets to which it was compared. According to Billeaud *et al.* (1982) there is a significant positive linear correlation between the osmolarity of the diet and the osmolarity in the stomach and duodenum. When the osmolarity of the digesta is still high when it reaches the end of the small intestine, it will retain water in the tract which can cause osmotic diarrhoea. Billeaud *et al.* (1982) conclude that diets high in osmolarity might be dangerous for low birth weight infants and should be avoided if possible. The results of this study show that the osmolarity of the Canine z/d® ULTRA Allergen Free diet is higher than the osmolarities of the other two diets at ingestion, but do not allow us to conclude this difference between the diets would persist at the end of the small intestine. In fact, the results of the work

in chapter three showed the Canine z/d® ULTRA Allergen Free diet has a high apparent ileal digestibility which implies that the osmotic effect of Canine z/d® ULTRA Allergen Free is unlikely to persist into the large intestine.

It is possible that the osmolarity of the Canine z/d® ULTRA Allergen Free diet was sufficiently high to directly damage the mucosa. To the author's knowledge this effect has only yet been reported at osmolarities higher than 410 mOsm/L (Kameda *et al.*, 1968; Norris, 1973). The highest osmolarity directly measured in this study was 350 mOsm in the Canine z/d® ULTRA Allergen Free diet at a weight/volume of 1:4. When the data was extrapolated into a weight/volume of 1:1 the osmolarity of Canine z/d® ULTRA Allergen Free was predicted to be approximately 679 mOsm/L. Although this osmolarity is higher than the 410 mOsm/L at which damage has been reported it is uncertain whether it is sufficiently high to cause damage. It is also possible the dilution of the hydrolysate by admixture with the gastrointestinal juices is sufficiently rapid that any exposure of the gastric or intestinal mucosa to high osmolarity would be transient. However, Billeaud *et al.* (1982) concluded that the osmolality of the two most hypertonic diets used in their study (about 600 mOsm/kg) remained high throughout the test (470 +/- 73 mOsm/kg at 45 min and 345 +/- 55 mOsm/kg at 180 min). This means there was a significant positive linear correlation between the osmolality of the diet and the osmolality in the stomach and duodenum at each time of sampling but the slope of the regression lines decreased progressively during the three hours after feeding.

Although one needs to be very careful drawing conclusions when extrapolating data, Billeaud's data and the data from the present study suggest that the hydrolysate diet was sufficiently hyperosmolar to be capable of damaging the mucosa.

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Chapter 5

Use of the breath hydrogen test to investigate if feeding Canine z/d® ULTRA Allergen Free is associated with carbohydrate malabsorption, small intestinal bacterial overgrowth and/or abnormal orocolic transit times

5.1 Introduction

Hydrogen gas (H_2), a by-product of microbial fermentation, can be measured in expired air and used as an indirect test for carbohydrate malabsorption, small intestinal bacterial overgrowth (SIBO) and/or abnormal orocolic transit times. Bacteria are usually found in high numbers in the colon and rectum. Colonic bacteria rapidly metabolise fermentable substrates that reach the large bowel and a proportion of the H_2 produced diffuses into the portal circulation and is subsequently excreted in the breath. The simple and noninvasive nature of the breath H_2 test and its ease of application to animals makes determination of breath H_2 concentration a suitable diagnostic test for small animal clinical practice (Bissett *et al.*, 1998).

Colonic flora produce H_2 while metabolising carbohydrate. Malabsorption of carbohydrates can occur because of events in the lumen of the intestine which interfere with normal digestive and absorptive processes (Rosenberg *et al.*, 1977). The two major causes of maldigestion are exocrine pancreatic insufficiency (EPI) and small intestinal disease (SID) (Williams and Guilford, 1996). The balance between the capacity of the colon to dispose of the carbohydrate by bacterial fermentation and the osmotic draw of the carbohydrate is probably the factor that determines whether carbohydrate malabsorption causes diarrhoea (Clausen, 1998).

The breath H_2 test relies on the fact that mammalian cells do not normally produce H_2 and therefore its presence in the breath indicates breakdown of carbohydrate in the intestine by bacteria. Because comparatively few bacteria live in the healthy small intestine, breath H_2 excretion does not usually occur to any great extent until malabsorbed carbohydrate begins to enter the colon, approximately 4-6 hours after the carbohydrate–containing food was ingested. Some of the H_2 will be absorbed into the blood and subsequently excreted by the lungs. Thus, after ingestion of a carbohydrate, a rise in breath H_2 indicates delivery of carbohydrate to the colonic flora (i.e., malabsorption), whereas no rise in breath H_2 indicates that the colonic bacteria did not gain access to carbohydrates (i.e., absorption). A linear relationship exists between the amount of carbohydrate malabsorbed and the amount of breath H_2 excreted (Bond and Levitt, 1972; Fritz *et al.*, 1985; Washabau *et al.*, 1986a).

The breath H_2 test has repeatedly been demonstrated to be an accurate indirect method of assessment of carbohydrate malabsorption and it has been widely applied in studying digestion of the entire spectrum of dietary carbohydrate (Perman, 1991; Muir *et al.*, 1991).

Small intestinal bacterial overgrowth (SIBO) is defined as an increase in the number of microorganisms in the lumen of the upper small intestine, in association with clinical signs of chronic diarrhoea and/or a malabsorption syndrome (Williams, 1996). SIBO is considered a significant cause of gastrointestinal signs in dogs (Williams, 1996; Rutgers *et al.*, 1996). Differences in diet, environment, age and breed of the animal, culture technique, and country of origin all contribute to the variation in numbers and types of bacteria in the small intestine (Johnston, 1999). Diet, in particular, has been proposed as a factor that can significantly influence the intestinal flora (Heine, 1996; Maciorowski, 1997) of dogs (Johnston, 1999).

In dogs with SIBO, the breath H_2 concentration rises early after feeding because the carbohydrate in the diet comes into contact with large numbers of bacteria in the small intestine that can ferment it far sooner after ingestion

than usual (Williams and Guilford, 1996). Thus, early rises of breath H_2 provide a method to diagnose small intestinal bacterial overgrowth. The breath H_2 test is viewed by some as the preferred method for testing for SIBO (Muir *et al.*, 1991; Stotzer and Kilander, 1999).

Another potential cause of diarrhoea after a meal is overly rapid transit through the small intestine. Decreased small intestinal transit times can be due to rapid gastric emptying. The shortened time of exposure of luminal contents to small bowel mucosa results in malabsorption and hence diarrhoea (Bond and Levitt, 1977). The rapid passage of food through the intestines also contributes to poor digestibility, high stool volume and gas production (Case *et al.*, 2000[a]).

The breath H_2 test has also been used for assessment of small intestinal transit times (Muir *et al.*, 1991). The time between feeding and first rise of breath hydrogen concentration is a measure of the time it takes for food to pass through the stomach and intestine to the colon – i.e. "orocolic transit time". The fidelity of this assessment can be upset if the small intestine (which usually has only small numbers of bacteria) becomes overgrown with large quantities of bacteria.

5.2 Objective of the study

The objective of the study was to assess if the Canine z/d® ULTRA Allergen Free diet causes carbohydrate malabsorption, small intestinal bacterial overgrowth and/or abnormal orocolic transit times by measuring the breath H_2 concentration in dogs after feeding the diet.

5.3 Materials and methods

5.3.1 Animals and diet

Ten healthy adult Labrador retriever-cross dogs (5 female, 5 male) with a body weight range of 26.5 to 34.6 kg were used in this study. The Animal Health Service Centre of Massey University supplied the dogs. The Massey University Animal Ethics Committee approved the experimental protocol.

The dogs' standard diet was Advance Pedigree Chicken[a], an AAFCO[b] complete and balanced dry diet. The proximate analysis and ingredients of Advance Pedigree Chicken are listed in table 5.1 and 5.2 respectively. The dogs were fed this diet at their maintenance energy requirement (MER) when they were not undergoing a breath hydrogen study. The test diets were Canine z/d® ULTRA Allergen Free[c] and Canine i/d®[d]. The amount of test diet fed prior to each breath hydrogen study was 50% of the dogs' MER. It was decided to restrict the test meal to 50% of the dogs' daily energy needs to help ensure the dogs ate the entire meal promptly, yet ensure sufficient dietary carbohydrate was ingested to challenge the dogs digestive and absorptive processes.

Table 5.1 Proximate analysis of Advance Pedigree Chicken

	%
Protein (not less than)	24.0
Fat (not less than)	14.0
Crude Fibre (not more than)	5.0
Moisture (not more than)	10.0
Ash (not more than)	10.5
Salt (as NaCL) (not more than)	2.5
Calcium (not less than)	1.2
Phosphorus (not less than)	1.2

[a] Waltham.
[b] Association of American Feed Control Officials.
[c] Hills Pet Products, Topeka Kansas.
[d] Hills Pet Products, Topeka Kansas.

Table 5.2 Ingredients of Advance Pedigree Chicken

Diet	Ingredients
Advance Pedigree Chicken	Chicken and chicken by-products, rice, corn, sorghum, chicken digest, chicken tallow, vegetable fibre, vegetable oil, vegetable protein concentrate, poultry and poultry by-products, iodised salt, potassium chloride, di-calcium phosphate, taurine, vitamin E, zinc sulphate, choline chloride, antioxidants, vitamin C, lucerne meal, marigold meal, tomato powder, ferrous sulphate (iron), copper sulphate, vitamin A, calcium pantothenate, sodium selenite, vitamin B2, vitamin B12, potassium iodide, vitamin B1, niacin, vitamin D3, vitamin B6, folic acid

5.3.2 Study protocol

Allotment of test diet (Canine z/d® ULTRA Allergen Free or Canine i/d®) to each dog was done at random (5 dogs were first fed z/d then i/d and 5 dogs were first fed i/d then z/d). The dogs were fasted for 24 hours prior to feeding of the test diet to reduce the effect of past meals on baseline breath H_2 results (see Figure 5.1 for complete outline of the study). The two test diets were fed for a maximum of 30 minutes after baseline breath H_2 sampling (T_0). The diets were fed to the dogs in a crossover repeated measure fashion because of the considerable intra- and inter-individual variation with the test (Ludlow, 1996 cited in Johnston, 1999). According to Bisset *et al.* (1998) there is no need for an adaptation period to the test diets.

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
8 am	Feed APC	Feed APC	Feed APC	T_0 [H2] collection	Feed APC	Feed APC	Feed APC	T_0 [H2] collection
			Fast	Feed z/d or i/d			Fast	Swap z/d or i/d
9 am				T_1 [H2] collection				T_1 [H2] collection
10 am				T_2 [H2] collection				T_2 [H2] collection
11 am				T_3 [H2] collection				T_3 [H2] collection
12 pm				T_4 [H2] collection				T_4 [H2] collection
13 pm				T_5 [H2] collection				T_5 [H2] collection
14 pm				T_6 [H2] collection				T_6 [H2] collection
15 pm				T_7 [H2] collection				T_7 [H2] collection
16 pm				T_8 [H2] collection				T_8 [H2] collection
17 pm	Feed APC	Feed APC		Feed z/d or i/d	Feed APC	Feed APC		Swap z/d or i/d

* APC = Advance Pedigree Chicken diet

Figure 5.1 Outline of study

5.3.3 Breath collection

The breath collection method used was that described by Washabau *et al.* (1986b). Briefly a close-fitting anaesthetic facemask was placed over the muzzle of the dog (Figure 5.2). The anaesthetic mask was connected to an anaesthetic reservoir bag via a non-rebreathing valve. To minimize dead space in the collection apparatus, the dogs were allowed to breathe through the system and fill the anaesthetic reservoir bag once without taking a breath sample. When the anaesthetic reservoir bag was filled it was removed, emptied and reattached while the dog continued to breathe through the facemask and non-rebreathing valve. After refilling the anaesthetic reservoir bag, an expired air sample was extracted from the base of the bag into a 20 ml syringe via a three-way stop valve. Plastic syringes with a rubber stopper were used as they represent practical vessels for storage of breath-test samples (Rosado and Solomons, 1983).

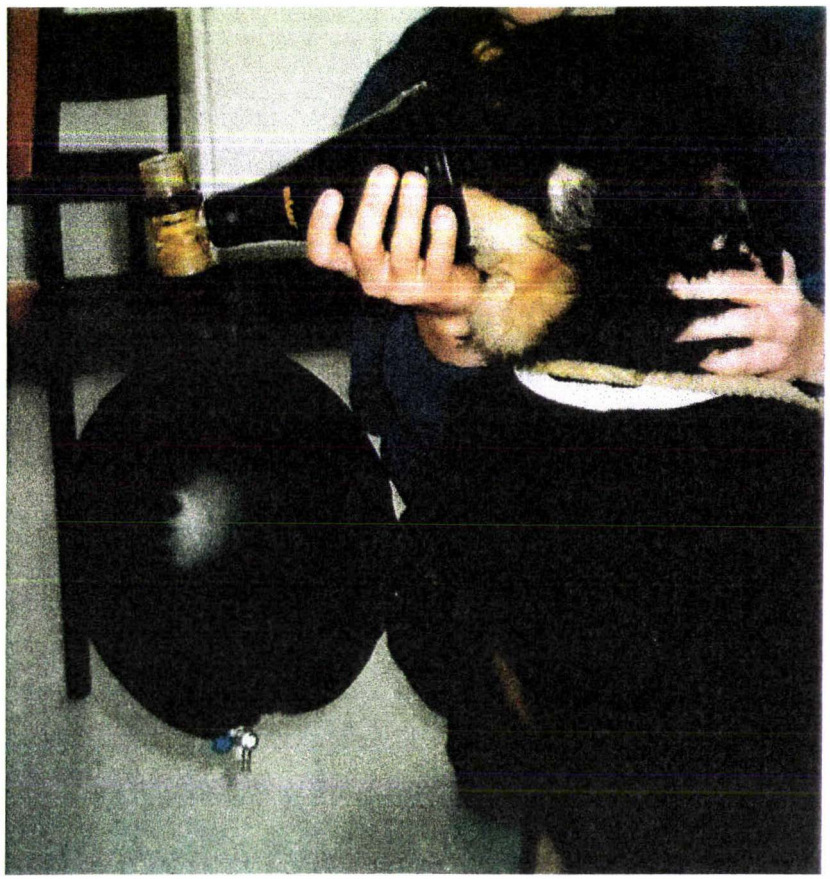

Figure 5.2 Anaesthetic facemask over muzzle of dog

Duplicate samples were obtained in order to detect sampling errors and to minimize the effect of large moment-to-moment variations in breath H_2. The dogs were carefully handled and exercised to minimise excitement during breath collections.

The samples were analysed within two hours of collection over which period there is no significant loss of H_2 concentration (Perman *et al.*, 1978 cited in Rosado and Solomons, 1983). An electrochemical cell[e] calibrated with a standard gas mixture was used for the analysis of H_2 concentration (ppm) in the breath samples. This meter has an accuracy of ± 1 ppm for the range of hydrogen concentrations measured in the trial (Muir *et al.*, 1991).

[e] GMI Exhaled Hydrogen Monitor, GMI Medical Ltd, Renfrew, Scotland.

5.3.4 Grading of faeces

Each dog's faeces were subjectively graded for consistency using a standardized grading chart. The grading began on Day 4 of the trial and was performed every time the dogs defaecated. A score of 1 to 5 was used, with 1 being a very watery stool and 5 representing a very firm stool.

5.3.5 Data analysis

The breath H_2 levels of the Canine z/d® ULTRA Allergen Free diet were compared to the breath H_2 levels of the Canine i/d® diet. H_2 measurements were analysed using the MIXED procedure in the SAS system. The model included the fixed effects of diet, time, the interaction of diet and time and the random effects of blocks of periods and dog nested within each diet. The residual covariance structure for repeated measures over time within dogs was assumed equal for all dogs. Least squares means and their standard errors were obtained for each combination of diet and time. The mean total area under the breath H_2 curves (AUC_{tot}) were calculated for both the Canine z/d® ULTRA Allergen Free and Canine i/d® diet. The mean of the maximum breath H_2 concentrations (peak breath H_2) and the time at which this maximum breath H_2 concentration occurred (time of peak breath H_2) were also determined for each diet.

The faecal grades of the dogs fed the Canine z/d® ULTRA Allergen Free diet were compared to the faecal grades of the dogs fed the Canine i/d® diet. This comparison was made using a paired T-test. The SAS[f] system was used for the statistical analysis of the faecal grade measurements.

[f] Release 8.02, 2001, SAS Institute Inc., Cary, NC, USA.

5.4 Results

All the animals remained healthy throughout the trial. The dogs consumed all their food readily.

The breath H_2 concentration of the Canine z/d® ULTRA Allergen Free and Canine i/d® diets were not significantly different from each other. Breath H_2 concentrations were significantly affected by time ($p<0.01$). The interaction of diet and time overall is not significant. This interaction is only significantly different at T5 ($p<0.01$). The breath H_2 concentrations of the Canine i/d® diet were significantly different ($p<0.01$) from the baseline breath H_2 concentration at T5 and T6. The breath H_2 concentrations of the Canine z/d® ULTRA Allergen Free diet were not significantly different from the baseline breath H_2 concentration.

The mean ±SEM breath H_2 concentrations of ten dogs over eight hours after the ingestion of the Canine z/d® ULTRA Allergen Free (z/d) and Canine i/d® (i/d) diet are presented in Figure 5.3.

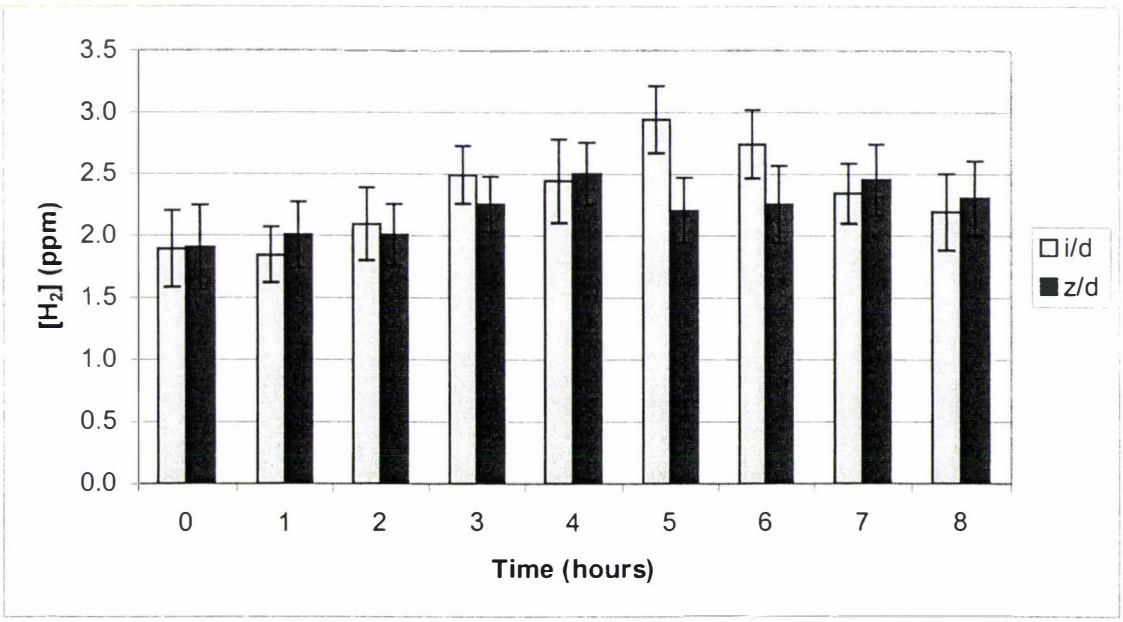

Figure 5.3 Mean ± SEM breath H_2 concentrations in 10 dogs after the ingestion of the Canine z/d® ULTRA Allergen Free and Canine i/d® diet

The mean ±SD areas under the breath H_2 curves (AUC), peak breath H_2 concentrations, and times of peak breath H_2 concentrations for the Canine z/d® ULTRA Allergen Free and Canine i/d® diet are displayed in Table 5.3. The area under the breath H_2 curve, the peak breath H_2 concentration, and the time of peak breath H_2 were not significantly different between the Canine z/d® ULTRA Allergen Free and Canine i/d® diet.

Table 5.3 Mean ±SD breath H_2 data after ingestion of the z/d or i/d diet

	AUC	Peak breath H2 (ppm)	Time of peak breath H2 (hours)
z/d	20.9321 +/- 1.8117*	3.2901 +/- 0.2195*	4.7 +/- 0.6839*
i/d	19.9679 +/- 1.8117*	3.1099 +/- 0.2195*	4.7 +/- 0.6839*

* not significantly different

There was considerable variation in the breath H_2 concentrations between individual dogs fed the test diet. This variation did not have the same pattern for every dog. When a dogs breath contains a higher H_2 with one diet at a given time, this doesn't necessarily mean that the dogs breath contains a higher H_2 at the same time with the other diet.

The mean ±SEM faecal grades of the Canine z/d® ULTRA Allergen Free and Canine i/d® diets were respectively 2.8 ± 0.25 and 4 ± 0.00. The faecal grades of the Canine z/d® ULTRA Allergen Free and Canine i/d® diets were significantly different ($p<0.01$) with Canine z/d® ULTRA Allergen Free having a significantly lower faecal grade.

5.5 Discussion

There was no difference between the mean breath H_2 concentrations of the dogs when fed the two test diets. These data also support that it is unlikely carbohydrate malabsorption explains the difference in faecal consistency between Canine z/d® ULTRA Allergen Free and Canine i/d®.

Although the breath H_2 concentrations in this study did not vary significantly between the two diets tested, they were observed to vary between dogs. Large individual variability in breath H_2 excretion after the ingestion of a test substrate has been reported in healthy dogs (Ludlow *et al.*, 1994), and is a well-recognized phenomenon in people (Bond and Levitt, 1972).

In this study all the dogs produced very little H_2 on both test diets. These low breath H_2 concentrations can be explained by the near complete digestion and absorption of the carbohydrates in both diets. When nearly all the carbohydrates in the diet are digested and absorbed there are no carbohydrates left for the colonic flora to produce H_2 while metabolising the carbohydrates. Perman (1991) showed low H_2 concentrations fluctuating around 10 ppm for healthy human patients fed a highly absorbable carbohydrate (rice starch) and Washabau *et al.* (1986a) showed H_2 concentrations fluctuating around 3 ppm and not exceeding 5 ppm in dogs fed xylose.

The results from this study agree with results found in Chapter 3 of this thesis. The results of that chapter show that the apparent ileal digestibility of the Canine z/d® ULTRA Allergen Free diet is high.
The orocolic transit time of the Canine i/d® diet – ie the time at which the breath H_2 concentration begins to increase – was approximately 5 hours which is normal. The results of the Canine z/d® ULTRA Allergen Free diet do not give such a clear message. There was no significant difference between baseline breath H_2 and any of the 8 time points. However, a recent paper by Ramirez *et al.*, (2003) concluded from a preliminary study that the osmolality

of the food does not change the gastric emptying in infants after feeding a higher osmolality food compared to a control food.

The lack of an early rise in breath H_2 excretion with the Canine z/d® ULTRA Allergen Free diet does not provide support for small intestinal bacterial overgrowth or rapid orocolic transit time.

The faecal consistency of the two test diets were significantly different from each other with the Canine z/d® ULTRA Allergen Free diet showing a more watery stool than the Canine i/d® diet. This is compatible with the higher than acceptable number of reports of diarrhoea Hill's Pet Nutrition received about the Canine z/d® ULTRA Allergen Free diet.

In conclusion, the results of this study suggest that the diarrhoea associated with Canine z/d® ULTRA Allergen Free diet is not due to carbohydrate malabsorption, small intestinal bacterial overgrowth or abnormal orocolic transit time. Nevertheless, the poor stool quality associated with the hydrolysate diet was readily observable but as yet remains unexplained.

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Chapter 6

Conclusion

Protein hydrolysates have come a long way in the food industry. While they were first used in hypoallergenic formulae for infants, they are now also used in diets for animals that are allergic to proteins. The original formulation of the Hill's Prescription Diet Canine z/d® ULTRA Allergen Free dog diet was associated with a higher than acceptable number or reports of diarrhoea. In this research report, three possible causes of this diarrhoea have been studied.

The first study was the assessment of apparent ileal digestibility of the Canine z/d® ULTRA Allergen Free diet using a rat model to determine if low digestibility may be contributing to the diarrhoea. This study showed that the digestibility of Canine z/d® ULTRA Allergen was high and it is therefore unlikely that poor digestibility contributes to the diarrhoea that has been reported in dogs fed this product.

The second study compared the osmolarity of Canine z/d® ULTRA Allergen Free to Prescription Diet Canine i/d® and Science Diet® Canine Maintenance. This study showed that the osmolarity of Canine z/d® ULTRA Allergen Free was approximately twice that of the osmolarities of Prescription Diet Canine i/d® and Science Diet® Canine Maintenance. This difference was statistically significant ($p<0.05$) and the data from this study suggest that the hydrolysate diet was sufficiently hyperosmolar to be capable of damaging the mucosa. However, it could not be concluded that this difference between the diets would persist throughout the entire length of the small intestine or that the diarrhoea associated with this diet was due to hyperosmolarity.

The last study assessed if the Canine z/d® ULTRA Allergen Free diet caused carbohydrate malabsorption, small intestinal bacterial overgrowth and/or abnormal orocolic transit times by measuring the breath H_2 concentration in dogs after feeding the diet. The faecal consistency of the two test diets were significantly different from each other with the Canine z/d® ULTRA Allergen Free diet showing a more watery stool than the Canine i/d® diet. There was no difference between the mean breath H_2 concentrations of the dogs when fed the two test diets. These data also support that it is unlikely carbohydrate malabsorption explains the difference in faecal consistency between Canine z/d® ULTRA Allergen Free and Canine i/d®. The look of an early rise in breath H_2 excretion with the Canine z/d® ULTRA Allergen Free diet does not provide support for small intestinal bacterial overgrowth or rapid orocolic transit time. Therefore it can be concluded that carbohydrate malabsorption, small intestinal bacterial overgrowth and rapid orocolic transit do not cause the diarrhoea in dogs fed the Canine z/d® ULTRA Allergen Free diet. The results from this study agree with results found in chapter 3 of this thesis. The results of that chapter show that the apparent ileal digestibility of the Canine z/d® ULTRA Allergen Free diet is high.

In conclusion, the results of the studies in this thesis have demonstrated that poor dietary digestibility, carbohydrate malabsorption, small intestinal bacterial overgrowth and abnormal orocolic transit times do not contribute to the occurrence of diarrhoea with this diet.

Further research into the osmolarity of Canine z/d® ULTRA Allergen Free during the whole process of ingestion through till defaecation is needed. It would also be valuable to determine if the diet produces any direct mucosal damage, by way of its relatively high osmolarity, or contains any secretagogues that may be causing excessive secretion of electrolytes and water. Lastly, the effect of the inclusion of a fermentable fibre on stool consistency, either through beneficial influence or colonic function or faecal water binding capacity should be investigated.

Appendix 1

Minimum and maximum nutrient levels of infant formulae
(National Research Council, 1989)

Nutrient	FDA 1971 Regulations Minimum	1976 Recommendations Minimum		Maximum
Protein (g)	1.8	1.8		4.5
Fat				
(g)	1.7	3.3		6.0
(% cal)	15.0	30.0		54.0
Essential fatty acids (linoleate)				
(% cal)	2.0	3.0		-
(mg)	222.0	300.0		-
Vitamins				
A (IU)	250.0	250.0		750.0
D (IU)	40.0	40.0		100.0
K (ug)	-	4.0		-
E (IU)	0.3	0.3	(with 0.7 IU/g linoleic acid)	-
C (ascorbic acid) (mg)	7.8	8.0		-
B (thiamine) (ug)	25.0	40.0		-
B2 (riboflavin) (ug)	60.0	60.0		-
B6 (pyridoxine) (ug)	35.0	35.0	(with 15 ug/g of protein in formula)	-
B12 (ug)	0.15	0.15		-
Niacin				
(ug)	-	250.0		-
(ug equiv)	800.0	-		-
Folic acid (ug)	4.0	4.0		-
Pantothenic acid (ug)	300.0	300.0		-
Biotin (ug)	-	1.5		-
Choline (mg)	-	7.0		-
Inositol (mg)	-	4.0		-
Minerals				
Calcium (mg)	50.0	40.0		-
Phosphorus (mg)	25.0	25.0		-
Magnesium (mg)	6.0	6.0		-
Iron (mg)	1.0	0.15		-
Iodine (ug)	5.0	5.0		-
Zinc (mg)	-	0.5		-
Copper (ug)	60.0	60.0		-
Manganese (ug)	-	5.0		-
Sodium (mg)	-	20.0		60.0
Potassium (mg)	-	80.0		200.0
Chloride (mg)	-	55.0		150.0