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UNIVERSITY OF SOUTHAMPTON

USE OF LACTOSE [$^{15}N^{15}N$]UREIDE TO QUANTIFY COLONIC SALVAGE OF UREA-NITROGEN

A thesis submitted for the degree of Doctor of Philosophy

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ABSTRACT

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USE OF LACTOSE [$^{15}N^{15}N$]UREIDE TO QUANTIFY COLONIC SALVAGE OF UREA-NITROGEN

by Rafe Bundy

The human body has a continual requirement for nitrogen in the diet as protein if form and function are to be maintained. The discovery that nitrogen is an essential component of the diet initiated research into protein, amino acid, and nitrogen *requirements*. The identification of an internal nitrogen salvage mechanism, whereby urea-N is made available to the body through the actions of the colonic bacteria, has opened a debate concerning the potential functional significance of this mechanism. However, a non-invasive method which can be used to quantify urea salvage has not been available.

Lactose-ureide was investigated as a potential means to deliver a known dose of ^{15}N label directly to the colon in order to non-invasively quantify urea salvage. Two lactose-ureides, labelled with either ^{13}C or ^{15}N on the urea moiety, were synthesized and analysed for purity. Following this, a clinical trial was conducted in which nine healthy adult individuals who had accommodated to a diet marginally adequate in protein were given oral doses of the lactose-ureides, and the excretion of label was followed. Results from the lactose [^{13}C]ureide administration demonstrated that little of the dose was excreted on breath before 6 h, with about 80 % undergoing fermentation and hydrolysis over a 48 h period, leading to the conclusion that lactose-ureide is a suitable vehicle to non-invasively deliver label to the colon intact. An analysis of urine and stool allowed a quantification of the fate of lactose [$^{15}N^{15}N$]ureide, demonstrating that about 5 % was excreted as urinary [$^{15}N^{15}N$]urea, 30 % as urinary [$^{14}N^{15}N$]urea, and 22 % in the stool fraction. It was concluded that, of the ^{15}N label available to the body, *over half* was retained on average. The discovery of label associated with lysine in the bacterial fraction of stool further suggests that *de novo* formed essential amino acids are available to the host. Additionally, a methodology was developed in order to directly address the potential functional significance of lysine formed *de novo* to the body, by attempting to separate the lysine fraction of apolipoprotein B-100 hydrolysates in a quantity sufficient to determine changes in ^{15}N enrichment using c-IRMS. An accurate detection of changes in ^{15}N enrichment as low as 0.002 APE in samples containing 3.3 ug lysine-N was achieved, and a distinction was made between the fate of lysine, lysine-N, and lysine-^{15}N through the analytical system.

A method has been developed which allows the salvage of urea-N to be non-invasively quantified in normal adults. Additionally, a methodology has been initiated which seeks to accurately determine changes in ^{15}N enrichment in lysine separated from plasma apolipoprotein B-100. These novel methodologies provide new tools with which to directly approach some of the unresolved questions surrounding human protein, amino acid, and nitrogen requirements.

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Now on to my next destination - Mars.

Cheers

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Preface

Nitrogen is essential for life. Although the earth's atmosphere consists of nearly 80% nitrogen, this must be fixed by organisms lower in the food chain before it can be utilized by humans. Humans have a continual *demand* or *requirement* for nitrogen which must be met by an adequate *supply* if form and function are to be maintained. The body may reutilize endogenous nitrogen, but when this is not sufficient to meet the demand, nitrogen must be obtained through the diet.

Nitrogen is present in the diet primarily in the form of protein. Millions of naturally occurring proteins are made up from combinations of 20 amino acids, all of which contain at least one amino group. The body utilizes protein in order to satisfy its metabolic demand for amino acids, which is set by the pattern of proteins being synthesized. The first limiting amino acid is the one in lowest abundance with respect to the metabolic demand. Other amino acids present in excess of the demand cannot be stored in the body and are degraded. The amino group is used predominantly to form urea, a nitrogenous product which can then be excreted.

Throughout history, there has been an innate interest in understanding the nutrient requirements of the body. For nitrogen, this interest has been pursued within an empirical framework since the scientific developments of the 18th century. When defining a requirement, it is important to ask - requirement for *what*? Outcome measures indicating satisfaction of the requirement need to be clearly and objectively identified. Initial studies investigated the *dietary* requirement for *protein*, measuring the intake of protein in the diet, and the loss of nitrogen in excreta. The outcome measure indicating that the requirement had been met was *nitrogen balance*, when intake and loss of nitrogen were equal. On a gross level, nitrogen balance measurements can provide valuable information, but there are inherent difficulties when trying to determine small changes in nitrogen status. Nor does the measurement of nitrogen balance give information on the internal control of nitrogen metabolism, exemplified by the observation that the body can attain nitrogen balance over a range of protein intakes.

The discovery and isolation of amino acids initiated the study of *dietary amino acid* requirements. Mixtures of artificially synthesized amino acids were fed to individuals, and the effects of omitting one or more amino acid were studied. Such experiments led to the classification of amino acids as either *essential* or *non-essential* in the diet, and dietary requirement values for individual amino acids were calculated. However, attaining nitrogen balance was still the outcome criterion upon which these values were calculated.

The use of stable isotopes in biological research has provided a means to follow the metabolic fate of individual amino acids, and to study human nitrogen requirements using different outcome criteria. Based on studies which have followed the metabolic fate of the carbon moiety of amino acids, dietary requirement values have been proposed which are up to two to three times higher than those calculated from nitrogen balance studies. These differences are important to address, not just for purely academic interest, but for the potential important implications for global food planning. In addition to these findings, the distinction between a *minimum dietary requirement* for an amino acid, which allows nitrogen balance, an *optimum dietary requirement*, which is beneficial to the individual, and a *metabolic* or *physiological* requirement for an amino acid, has been highlighted.

Since the discovery in the late 1950's that urea is continually hydrolyzed in the colon of humans through the activity of the resident bacteria, there has been interest in the utilization of nitrogen made available to the body through this *salvage* mechanism. It has been demonstrated that as dietary protein intake falls from 70 to 35 g/d, urea salvage increases, and a portion of the nitrogen is retained by the body. This suggests a functional significance, but it is not clear in what form the nitrogen is retained. Although host utilization of amino acids synthesized *de novo* by the resident bacteria is well established in ruminant nutrition, there has only been limited data to support this in humans. The relative inaccessibility of the colon has made it difficult to obtain quantitative data on the fate of hydrolyzed urea in normal individuals. The aim of the thesis is to develop a novel non-invasive technique which can be used to quantify the fate of salvaged urea in normal individuals. It is hoped that this will provide a new tool to help contribute understanding to some of the outstanding questions surrounding human protein, amino acid, and nitrogen requirements.

DR RANCE	*The vehemence of her denials is proof positive of guilt. It's a text-book case! A man beyond innocence, a girl aching for experience. The beauty, confusion and urgency of their passion driving them on. They embark on a reckless love-affair. He finds it difficult to reconcile his guilty secret with his spiritual convictions. It preys on his mind. Sexual activity ceases. She, who basked in his love, feels anxiety at its loss. She seeks advice from the priest. The Church, true to Her ancient traditions, counsels chastity. The result - madness!*
DR PRENTICE	*It's a fascinating theory, sir, and cleverly put together. Does it tie in with the known facts?*
DR RANCE	*That need not cause us undue anxiety. Civilizations have been founded and maintained on theories which refused to obey facts.*

from *'What the Butler Saw'*
by Joe Orton

CHAPTER 1

Literature Review

1.1 Nitrogen : a historical perspective

The review of literature opens by considering how nitrogen was viewed in nutrition up until the discovery and isolation of amino acids at the turn of the 20th century. Even before nitrogen was characterised, it had been noted by researchers interested in the body and its function that a necessary dietary component was contained in *'the gelatinous lymph'* of animals, and in the *'small portion of jelly'* of vegetables (Carpenter, 1994). This was followed by the recognition of a continual need for nitrogen containing compounds in the diet to maintain life. Since the primary form of nitrogen in the diet is protein, this lead to initial proposals for a dietary requirement of protein for individuals.

Daniel Rutherford discovered nitrogen in 1772. He noted that an animal left to respire in a closed container would eventually die, and that the remaining air could not support life. After the removal of carbon dioxide, which had been characterised by his mentor Joseph Black, Rutherford termed the residual gas *aer malignus* (noxious air). This was given the name *nitrogen* in a translation of

a French chemistry book 20 years later. Antoine Lavoisier, a French chemist, was interested in the work of Black and Rutherford, and conducted the first documented metabolic experiment with nitrogen in 1790 (Munro, 1964). Lavoisier observed that no nitrogen was lost from the body during respiration, and therefore concluded that gaseous nitrogen in the air plays no part in body nitrogen metabolism. This drew a distinction between organic and inorganic nitrogen, and methods to analyse the organic nitrogen content of materials were subsequently developed by Lavoisier's students, among them Gay-Lussac. Lavoisier studied the composition of isolated plant fractions and concluded that sugars, gums and starches consisted only of combinations of carbon, hydrogen and oxygen, whereas Claude Berthellot had shown in 1785 that animal tissue also contained nitrogen (Carpenter, 1994).

Adopting the solid chemical viewpoint laid down by Lavoisier, Francois Magendie made a clear distinction between the nutritive value of nitrogenous and non-nitrogenous dietary components. From a series of feeding experiments, he concluded that a nitrogen source was essential in the diet, since dogs would not survive more than 40 days without dietary nitrogen. In an attempt to explain how herbivorous creatures and certain groups of people could survive whilst consuming no meat in the diet, he wrote in a textbook: *'almost all the vegetables upon which man and animals feed contain more or less azote (nitrogen)'* (1829). He was also the first to suggest a continuous turnover of bodily constituents which proceeded at different rates depending on the tissue or organ in question, a dynamic view of nutrition which was not explored further until more than a century later. Magendie, along with two other French scientists, noted differences in the quality of different nitrogen containing foodstuffs. Puppies fed diets consisting of bread and gelatin - the *animal substance* obtained from cooking bones - would not grow as well as those provided with meat bouillon instead. This lead Jean Baptiste Boussingault to measure the nitrogen content of various plant-derived foods and suggest that their nutritional value was proportional to their nitrogen content, even though some of Magendie's results did not support this rather basic viewpoint (Carpenter, 1994).

Justus von Liebig studied chemistry in Paris between 1823 and 1824 and then returned to his native Germany where he set up the most renowned school of organic chemistry of the time (Munro, 1964). Turning his knowledge to the study of the living organism, as Magendie had done, he recognised different types of nitrogen containing compounds in both animal and plant matter, giving the names *fibrine* and *albumen* to the two chief ones. He stated that these two compounds are essentially made from the same chemical constituents and can be incorporated into muscle and subsequently reconverted into blood. Liebig portrayed the important role played by nitrogen in the body: *'All part of the animal body which have a decided shape, which forms part of organs, contain nitrogen ...The most convincing experiments and observations have proved that the animal body is absolutely incapable of producing an elemental body, such as carbon or nitrogen, out of substances which do not contain it; it obviously follows, that all kinds of food fit for the production either of blood, or of cellular tissue, membranes, skin, hair, muscular fibre, etc. must contain a certain amount of nitrogen because that element is essential to the composition of the above named organs; because the organs cannot create it from other elements presented to them; and, finally, because no nitrogen is absorbed from the atmosphere in the vital process'* (1842). Gerald Mulder, a Dutch chemist, had put forward a theory in 1838 suggesting a common nitrogenous sub-unit contained within organic nitrogen-containing compounds. This was linked to phosphorus and sulphur in molecules to form the various nitrogenous compounds already discovered. The name *protein* - from the Greek word *proteios* meaning 'of the first rank' - was suggested for this new radical by the Swedish chemist Berzelius, *'...because it appears to be the fundamental or primary substance of animal nutrition which plants prepare for herbivores, and who in turn supply it to carnivores'* (Vickery, 1950). This theory was endorsed by Liebig in his first edition of *Animal Chemistry*, and by 1842 it was generally accepted that all nitrogenous components in animal and plant tissues were based on a common large unit called protein. Liebig went even further by suggesting that the protein radical was in fact the only true nutrient essential for both body building and physical activity, while the non-nitrogenous components of the diet merely supported the process of respiration (Carpenter, 1994). A number of scientists criticised this view, resulting in Liebig completely abandoning the protein theory

when he was unable to reproduce Mulder's original experiments. However, he still maintained that only the 'albuminoids' in plant and animal matter were of nutritional significance and provided the body with mechanical energy. It is ironic that the current definition of a protein, a substance containing one or more chains of amino acids, covers such an immense range of organic molecules rather than a single unit as first proposed by Mulder.

The discovery of nitrogen as such an essential dietary component obviously led to investigations into the dietary requirement for nitrogen. One of the earliest methods developed was the measurement of *nitrogen balance*. The body is said to be in nitrogen balance when the intake of nitrogen (mainly as protein in the diet) equals the output of nitrogen (in the urine, stool, and other bodily excretions). This technique has been widely employed by many investigators over more than 150 years, and has also been used to determine the adequacy of nitrogen in particular diets. Boussingault can be regarded as the first to attempt such a balance study when he measured carbon, hydrogen, oxygen and nitrogen intake and output of a cow (1839*a*) and a horse (1839*b*). He observed that the animals excreted less nitrogen in milk, urine and faeces than they consumed, and suggested that the discrepancy was due to the excretion of nitrogen from the lungs until Liebig ridiculed this hypothesis. It was Liebig's student, Carl Voit, who further refined the nitrogen balance technique. Voit noted that dogs required a certain amount of nitrogen in their diet, as meat, to maintain nitrogen balance. If less meat was given, excretion of nitrogen in the urine and faeces would exceed dietary intake. After further studies, Voit concluded that nitrogen intake and output were equal in healthy subjects over the long term, producing a state of nitrogen equilibrium (Carpenter, 1994).

In the middle of the 19th century, the British scientist Playfair set out a list of dietary requirements for protein ranging from 57 to 184 g/d depending on the physical activity of the individual, since Liebig's theory that proteins provided the only source of mechanical energy was still widely accepted at this time (Munro, 1964). Voit summarised the results of his own research and that of his predecessors in 1881 and suggested a requirement of 118 g protein/d - one half to come from animal sources - for an *'average worker'*, being a necessarily

well-muscled man. This view was supported by his student, Atwater, who proposed a US standard of '*125 g for a man at moderate muscular work*' in 1891. However, another school of thought proposed lower intakes of protein. This idea was originally carried forward by the Romantic movement of the early 19th century whose abhorrence of killing led the participants toward the consumption of vegetarian diets. These views were adopted by religious movements such as the Seventh Day Adventists who, led by figures such as Sylvester Graham and John Harvey Kellogg, warned of *'overstimulation'* of the body with a high protein diet. This argument was developed in a more scientific framework by Russell Chittenden at the end of the 19th century. Chittenden challenged Atwater's recommendations and demonstrated that an intake of 50 to 55 g protein/d was sufficient to maintain nitrogen balance regardless of physical activity, and actually increased the physical strength, health, and general well-being of his subjects (1905). Another researcher, Siven, had proclaimed that balance could be maintained on as little as 30 g protein/d (1901). Critics questioned whether this was only a short-term phenomenon, and maintained that such a diet compromised the *'productive power'* of workers. They argued that poor diets were only as a result of poor income, whereas Chittenden's view was that the affluent West was over-eating protein.

It can be seen that although investigators agreed that a dietary source of nitrogen was essential for life, opinions were divided on how much protein was suitable or adequate in the diet of individuals. Liebig's hypothesis that protein was the only source of muscle energy had been categorically disproved in 1865 by two Swiss investigators, Fick and Wislicensus, but his views still persisted in the minds of many researchers. The issue of dietary protein requirements was further confounded by the observation that nitrogen balance could be apparently maintained over a wide range of intakes. The idea that a difference in the quality of dietary protein could be a part of this apparent conundrum was investigated further after the discovery and isolation of amino acids.

1.2 The discovery and classification of amino acids

The recognition of amino acids as structural components of proteins opened the way for new concepts of the nutritive value of proteins. Braconnot had first observed amino acids as the products of acid hydrolysis of protein in 1820 but, along with others investigators like Liebig, he deemed that they were not of any biological significance due to the extreme nature of protein breakdown which was needed to produce them (Munro, 1964). Over the next 50 years, there was increase in the knowledge relating to the digestion of proteins in mammals, and pancreatic juice was shown to degrade protein with the subsequent recovery of the amino acids leucine and tyrosine (Carpenter, 1994). However, researchers of the time were still not convinced that amino acids were the sub-units constituting proteins since yields from protein hydrolysates corresponded to less than 50% of the original weight of the protein. A theory was proposed that amino acids were in fact formed in the gut from proteins ingested in excess of bodily requirement, which were then absorbed, converted to urea, and excreted (Lea, 1890).

By 1900, Kossel and Kutscher had developed a method which allowed quantitative separation of the basic amino acids from protein hydrolysates. The earlier suggestion by Ritthausen (1872) that differences in the quantities of individual amino acids may influence the nutritional value of proteins, which he had observed in plant and albumin hydrolysates, was now explored further. Willcock and Hopkins showed in 1906 that mice fed the protein zein, which had been shown to be deficient in the amino acid tryptophan, lost weight and survived only half as long as those supplemented with additional tryptophan. Osbourne and Mendel later found that young rats could maintain their weight for a few weeks if supplemented only with tryptophan, but would not grow unless supplemented with tryptophan and lysine (1914). It was now widely accepted that establishing which amino acids were indispensable dietary components was of great importance in the study of protein metabolism. By 1932, only three amino acids had been shown to be indispensable in the diet of the growing rat, but the discovery and identification of the amino acid threonine a few years later

made it possible for the first time to investigate nitrogen requirements by feeding diets where the sole nitrogen source was provided as amino acids.

Rose and his colleagues were pioneers in the field of body protein metabolism, investigating both the qualitative and quantitative aspects of dietary amino acids. Growing rats were fed different diets where nitrogen was provided as a mixture of purified amino acids, and it was observed that only ten of the twenty amino acids typically found in proteins were necessary for maximum growth (1948). Rose and his co-workers then went on to repeat these experiments in adult males, using nitrogen balance as the criterion of nutritional adequacy in each subject (1957). Mixtures of the ten essential amino acids needed for rat growth were administered in the diet, providing between 6.7 and 10.0 gN/d. Adequate energy and a vitamin supplement were also given. After a period of a few days, when nitrogen equilibrium was maintained, a single amino acid was omitted from the diet while the total nitrogen was kept constant by proportionally increasing the amounts of the other amino acids. The results of these studies led Rose to concluded that eight amino acids were essential in the diet of adult men to maintain nitrogen equilibrium (Table 1.1). Rose defined an essential dietary component as *'one which cannot be synthesised by the species in question...at a rate commensurate with the needs for optimum growth'*.

More recent investigations of amino acid metabolism in the body have led to the need to expand the definitions proposed by Rose. For example, some non-essential amino acids, such as tyrosine and cysteine, can become essential in conditions where there is a lack of the dietary precursor molecule, phenylalanine and serine respectively. Similarly, the carbon skeleton of many essential amino acids (leucine, isoleucine, methionine, phenylalanine, tryptophan and valine) can be aminated endogenously. Jackson (1983) has subdivided amino acids into four main groups, reserving the term *essential* for those amino acids whose carbon skeletons cannot be synthesised or aminated and so must be supplied by the diet (lysine and threonine). Conversely, the carbon skeletons of the amino acids which he defines as *non-essential* (alanine, glutamic and aspartic acid) can be both synthesised and aminated endogenously (Table 1.2). In the same year, Harper made the point that the term

non-essential was misleading, since it suggests that these amino acids are never required in the diet, and therefore proposed that the term *dispensable* be used instead.

Table 1.1 Classification of amino acids according to their role in the maintenance of nitrogen equilibrium in normal young men (after Rose, 1957)

ESSENTIAL	*NON-ESSENTIAL*
Valine	Glycine
Leucine	Alanine
Isoleucine	Serine
Threonine	Cysteine
Methionine	Tyrosine
Phenylalanine	Aspartic Acid
Lysine	Glutamic Acid
Tryptophan	Proline
	Hydroxyproline
	Histidine
	Arginine
	Citrulline

Table 1.2 A new classification of amino acids (after Jackson, 1983).

AMINATION OF CARBON SKELETON	SYNTHESIS OF CARBON SKELETON	
	YES	*NO*
YES	Non-essential amino acid	Essential carbon skeleton
NO	Semi-essential amino acid	Essential amino acid

Laidlaw and Kopple (1987) have also reclassified dietary amino acids, dividing them into five categories. *Totally indispensable, carbon-skeleton indispensable,*

conditionally indispensable and *dispensable* all closely correlate with the divisions proposed by Jackson. The fifth category, *acquired indispensable*, is used to define amino acids which can become indispensable in states of disease (such as chronic liver failure), immaturity of synthetic processes (such as in premature birth), and severe metabolic stress.

After the isolation of amino acids had been made possible, the meticulous work of Rose and his colleagues allowed for the first comprehensive qualitative definition of the twenty amino acids present in the human diet. During this time, Block and Mitchell (1946) were able to show that the essential amino acid content of a protein, expressed as an analytically derived 'chemical score', provides an indication of the biological value of that protein for man. Rose continued his investigations in a quantitative manner, leading to the first published dietary recommendations for the eight 'essential' amino acids he had defined.

1.3 Dietary essential amino acid requirements based on studies of nitrogen balance

In determining dietary requirement values for the eight essential amino acids, Rose's objective was to establish the *minimum* intake of each essential amino acid which would permit nitrogen equilibrium when the diet contained sufficient quantities of the other seven essential amino acids *plus* a supply of nitrogen adequate for the synthesis of the non-essential amino acids (Rose, 1957). It is important to note the need to supply non-essential nitrogen in these and other studies of dietary requirements in order that nitrogen equilibrium be attained, since it highlights the distinction between dietary requirements for essential amino acids and a dietary requirement for total nitrogen. Non-essential nitrogen can be defined as additional sources of dietary nitrogen other than the essential amino acids, and these include: essential amino acids in excess of the metabolic requirement; dispensable amino acids; and other nitrogenous compounds such as urea and ammonium salts. The diets administered in the quantitative studies of Rose contained a total nitrogen intake for each subject of

approximately 10.0 gN, consisting of the eight essential amino acids plus additional glycine and urea for the synthesis of non-essential amino acids. The amino acid under study was progressively withdrawn from the diet while the concentration of the other essential amino acids was kept constant, the shortfall in nitrogen made up with glycine. The conclusions drawn from these studies are shown in Table 1.3. Rose noted individual variation in the intake for each amino acid which would permit nitrogen equilibrium, and subsequently proposed two levels of intake - a *minimum* amount which was equivalent to the highest value measured, and a *safe* level of intake which was twice the highest value measured and which should meet the dietary needs of any normal adult.

Table 1.3 The daily amino acid requirements of young men (after Rose, 1957)

Amino Acid	*Range of recorded requirements (g)*	*Minimum proposed intake (g)*	*Definite safe intake (g)*
Tryptophan	0.15-0.25	0.25	0.50
Phenylalanine	0.80-1.10	1.10	2.20
Lysine	0.40-0.80	0.80	1.60
Threonine	0.30-0.50	0.50	1.00
Methionine	0.80-1.10	1.10	2.20
Leucine	0.50-1.10	1.10	2.20
Isoleucine	0.65-0.70	0.70	1.40
Valine	0.40-0.80	0.80	1.60

Rose subsequently showed that the safe level of intake was adequate to maintain nitrogen equilibrium in all the subjects who were studied, although he was careful to point out that *'...safe must not be considered synonymous with optimum'*, a consideration which will be discussed later.

Dietary requirements for the eight essential amino acids were also assessed for adult women and published by Leverton and her colleagues (Leverton, 1959). Their results suggested minimum dietary requirements consistently lower than those proposed by Rose. One important difference to note in the

methodology employed by the two groups which may help to explain these variations, is the definition of nitrogen equilibrium. Leverton accepted nitrogen balance as *'the zone in which the difference between the intake and excretion does not exceed 5%, i.e. the excretion is within 95 to 105 % of the intake'* which differed from subjects studied by Rose attaining *'a distinctly positive balance, as measured by the average of a period of several days'*. This difference highlights one of the limitations of using the nitrogen balance technique to assess dietary amino acid requirements. Two large values (nitrogen intake and nitrogen output) are subtracted from each other to determine the state of nitrogen balance, and even small differences in these measured values will result in a proportionately greater variation in the minimum amount of each essential amino acid which is deemed adequate in the diet.

1.4 Limitations of the nitrogen balance technique

It is worth considering some important observations that have been made during nitrogen balance studies, and the subsequent limitations of using the nitrogen balance technique to determine dietary essential amino acid requirements. The first relates to the loss of body nitrogen other than through the urine and stool, termed miscellaneous losses. The experiments of Rose and Leverton had taken no account of any of these losses in their calculations of nitrogen balance (Fuller and Garlick, 1994). However, small differences in the loss of nitrogen through these routes could be sufficient to change apparent nitrogen balance from a negative to a positive value. A series of studies funded by National Aeronautics and Space Administration in the 1960's were conducted to quantify miscellaneous losses of nitrogen by accurately measuring all known routes of nitrogen output from the body, including sweat, skin, and saliva. It was concluded that the total error in nitrogen balance studies taking no account of miscellaneous losses could be as high as 0.5 gN/d, and that exercise-induced sweating and increasing protein intake greatly increased dermal losses of nitrogen (Calloway *et al*, 1971). Using a conservative estimate for miscellaneous losses of nitrogen, Fuller and Garlick have recently recalculated the values for dietary essential amino acids needed to attain nitrogen

equilibrium in females, as reported by Leverton, and suggested amounts up to 2.5 times greater (1994), although these values still fall within the *safe* intakes for an individual as proposed by Rose (1957). Despite taking these losses into account, investigators have observed wide individual variation in total nitrogen excretion between subjects (CV 18 % in urinary nitrogen between subjects on a virtually nitrogen-free diet), explained as being due to individual differences in protein catabolism (Carpenter, 1994).

The second observation relates to the effect of energy intake on measured nitrogen balance. Rose had noted that a high intake of energy, up to 58 kcal/kg/d, was needed by many of his subjects to achieve nitrogen balance when fed diets consisting of mixtures of essential amino acids (1957). Other groups have been unable to achieve nitrogen balance using the same amount of nitrogen as Rose (9 g as a mixture of the eight essential amino acids and 27 g as glycine) when energy intake was lower, for example 45 to 46 kcal/kg/d (Kies *et al*, 1965). However, this protein-sparing effect of increased energy intake does not mean that energy can be used in the diet to substitute for an essential amino acid. As Rose pointed out, *'...no calorie intake, however, large, is capable of inducing nitrogen balance when the food is deficient in a single amino acid'* (1957).

A further observation relates to the total nitrogen intake in the diet. As previously mentioned, enough *non-essential nitrogen* has to be given in the diet along with the mixture of essential amino acids to maintain nitrogen balance. This is to allow for the endogenous synthesis of dispensable amino acids. It had been observed that the total daily requirement for essential amino acids was only 16 % of the total protein intake needed to maintain nitrogen balance (Carpenter, 1994). Since essential amino acids make up about 45 % of body protein, it had previously been assumed that the diet would have to contain a similar proportion of amino acids in order to maintain body tissues. Over the years, researchers have noted that nitrogen balance can be improved when non-essential nitrogen is added to the diet. For example, Snyderman *et al* (1962) observed that three infants fed diets containing between 18-26 mgN/kg as cow's milk failed to gain weight, but would continue to grow when

supplemented with nitrogen in the form of glycine or urea. Similarly, Tripathy *et al* (1970) demonstrated that urea supplementation induced a significant positive nitrogen balance in 10 malnourished adults in negative nitrogen balance consuming low protein diets. Kies and co-workers have used the results from their many studies of nitrogen balance to develop a rank order of the effectiveness of different sources of non-essential nitrogen to supplement low protein diets. This has been characterised by Jackson (1995) as a system of nitrogen exchanges, such that the closer the pattern of amino acids in the diet to the metabolic demand for amino acids, the less total amount of dietary amino acids required. However, as with energy, it is important to note that additional nitrogen will only improve nitrogen balance when the minimum amount of each essential amino acid needed to satisfy the metabolic demand is present in the diet. Non-essential nitrogen cannot be used to substitute for essential amino acids.

It has been shown in this section that the nitrogen balance technique has a number of inherent limitations which can make interpretation of the results difficult. Specifically, the apparent minimum dietary requirement for an essential amino acid needed to maintain nitrogen equilibrium can be influenced by how miscellaneous losses of nitrogen are accounted for, and the amount of energy and non-essential nitrogen consumed with the diet. The subsequent use of stable isotopes in biological research has allowed for the study of protein, amino acid, and nitrogen requirements using different markers of nutritional adequacy, and this has transformed much of our understanding in the area of human nitrogen metabolism.

1.5 The advent of stable isotopes in nutrition

A major advance in the study of protein, amino acid and nitrogen metabolism came with the development of techniques using stable isotopes, which were identified during the 1920's and 30's (Carpenter, 1994). Over 99 % of naturally occurring nitrogen is in the form of ^{14}N, but a small proportion, about 0.37 %, occurs as the stable isotope ^{15}N. This form of nitrogen contains an additional

neutron, but has the same pattern of electrons in orbit around the nucleus. Therefore, ^{15}N has the same chemical and biological properties as the most abundant form of the element, but has a different physical character which can be used as an analytical means of identification and separation. Once physicists and chemists had discovered ways to prepare materials with a high proportion of stable isotopes, biological experiments could begin (Carpenter, 1994). Rudolf Schoenheimer is widely recognised as the pioneer of stable isotope work in the area of nutrition.

After Liebig's theory that protein was the sole source of muscular energy was disproved by a number of investigators, Voit went on to develop his own view of protein metabolism. He observed in dogs that there was a positive correlation between the excretion of urinary nitrogen during the first few days of a fast, and the amount of protein present in the preceding diet. By 1867, Voit had gone on to propose a labile pool of 'storage' or 'circulating' protein in the body, the size of which was dependant on the level of protein intake. Folin later suggested a theory which separated protein metabolism into two independent compartments - a relatively constant endogenous or tissue metabolism, and a variable exogenous metabolism. This was based upon the observation that a change in protein intake affected that output of some urinary components, such as urea, but not others (1905). It was Schoenheimer who first discredited Folin's theory of compartmentalised protein metabolism. Schoenheimer and his colleagues (1939) administered the amino acid leucine, labelled with ^{15}N and deuterium, ^{2}H, to mature rats on an adequate protein diet. From the deuterium content of leucine isolated from the rat, it was calculated that 6.7% of the carcass leucine was derived from leucine consumed over 3 days during the study. These results indicated that the rate of protein synthesis was in fact three times greater than that calculated from the endogenous loss of nitrogen in similar rats consuming nitrogen free diets. This was in striking contrast to the commonly held belief that protein breakdown, and therefore synthesis, proceeded at the same rate as the loss of nitrogen from the body. Schoenheimer noted from following the fate of ^{15}N label that there existed a considerable active exchange of amino groups from leucine to other amino acids in the body, notably glutamic acid, and it was also demonstrated that the rapid appearance of label in body protein was

widespread among other amino acids. In *The Dynamic State of Body Constituents*, published in 1942, Schoenheimer theorised that there was continual synthesis of amino acids, and metabolic exchange with body proteins, *'...even when there is no obvious need for it.'*

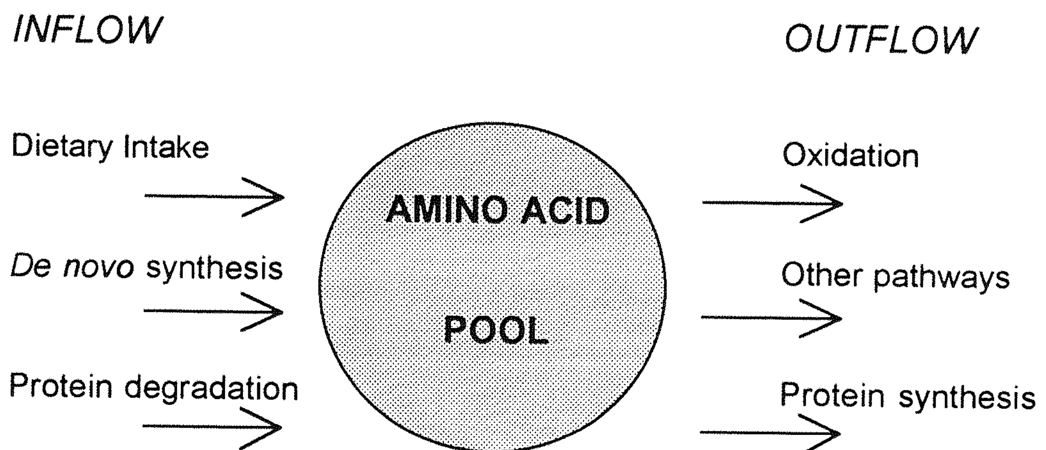
Schoenheimer's work has transformed the way in which protein metabolism is viewed conceptually, even though the significance of his findings were not widely regarded at the time (Carpenter, 1994). His contribution has led to the development and use of stable isotope methodologies to trace the metabolic fate of protein, amino acids, and nitrogen in the body. The next section will consider how such methodologies have been employed more recently as a means to determine essential amino acids requirements, other than by using the nitrogen balance technique.

1.6 Use of the tracer balance technique to determine adult essential amino acid requirements

The nitrogen balance technique has a number of inherent limitations which have been discussed in the previous section. Stable isotope techniques have subsequently been employed to re-evaluate the requirements for essential amino acids in humans. This section will describe the contribution of the ^{13}C tracer balance technique, the conclusions which have been drawn from some of the studies, and the assumptions upon which these conclusions are based. First, it is important to consider the general model which is used to determine body amino acid kinetics (Figure 1.1). An amino acid flows through a single amino acid pool which is metabolically regulated. Since the body cannot store free amino acids in high concentrations, the size of the pool is small relative to the daily flux. The flux or flow through any amino acid pool is made up of three *inflows* and three *outflows*. Amino acids enter the pool from the digestion of dietary protein, the degradation of endogenous protein, and *de novo* synthesis. The outflows are comprised of amino acids which are irreversibly oxidised during degradation, used in the synthesis of endogenous protein, and metabolically consumed in pathways other than protein synthesis (Jackson,

1995). By administering isotopically labelled amino acids to the body, it is possible to obtain information on the kinetics of amino acids. In general terms, information is obtained by observing the *dilution* of labelled amino acids within the pool, and the *rate* at which labelled amino acids exit the pool.

Figure 1.1 The general model for the kinetics of an amino acid (after Jackson, 1995)



The ^{13}C tracer balance method is a stable isotope technique which has been extensively used to estimate adult essential amino acid requirements by Young and his colleagues since the early 1980's. The principle behind the method is as follows: amino acids consumed in excess of the metabolic demand cannot be stored, and so are irreversibly oxidised. This means that after removal of the nitrogen containing amino group, the carbon skeleton of the amino acid is oxidised to release CO_2 and energy. Since there is a minimum loss of amino acids through oxidation, called the OOL (obligatory oxidative loss), the amino acid intake of an individual which is equal to the OOL can be defined as the *minimum dietary requirement* for that amino acid (Young *et al*, 1989).

The general procedure employed during ^{13}C tracer balance studies is to administer amino acids labelled with ^{13}C to individuals, and measure the excretion of the label as $^{13}CO_2$ in the breath and the dilution of labelled amino acid in the plasma. From these measurements, the amino acid oxidation rate is calculated. Based on the interpretation of their findings, Young *et al* (1989) have proposed an increase to the current values for adult dietary essential amino

acid requirements, as recommended by the Food and Agriculture Organisation/World Health Organisation/United Nation University Expert Consultation (FAO/WHO/UNU, 1985), which they have called the Massachusetts Institute of Technology (MIT) pattern. These values are compared in table 1.4.

It is important to address the issues raised by the large differences in values for essential amino acids proposed by Young's group compared with those currently recommended by the FAO/WHO/UNU (1985). There are world-wide agricultural and economic implications to consider, since an analysis of the diets consumed in developing countries has led to the conclusion that, based on Young's proposals, lysine is likely to be limiting in cereal based diets. Consequently, for an individual to have an intake that meets the minimum MIT value, about 40 % protein intake will have to come from animal sources or legumes (Young and Pellet, 1990).

Table 1.4 The MIT pattern of essential amino acid requirements in healthy adult humans (after Young *et al*, 1989) compared with current FAO/WHO/UNU (1985) recommendations.

AMINO ACID	*MIT Pattern**	*FAO/WHO/UNU Recommendation**	*Increase+*
Isoleucine	23	10	x 2.3
Leucine	40	14	x 2.9
Lysine	30	12	x 2.5
Methionine and Cysteine	13	13	-
Phenylalanine and Tyrosine	39	14	x 2.8
Threonine	15	7	x 2.1
Tryptophan	6	3.5	x 1.7
Valine	20	10	x 2.0

* Values expressed as mg/kg body weight/d

+ Values expressed as fold increase compared to the current recommendations

The theoretical model employed by Young has its own inherent limitations, and some of the conclusions drawn from his tracer balance studies have been questioned by a number of investigators. When assessing the dietary requirement for an essential amino acid, Young assumes that the inflow to the amino acid pool from *de novo* synthesis is zero, and that the outflow to other metabolic pathways is small enough to be ignored (Jackson, 1995). These two assumptions need to be considered carefully. Fuller and Garlick (1994) have noted the possible routes of amino acid loss to metabolic pathways other than oxidation, and estimated the percentage of daily requirement which may be lost through these routes (Table 1.5).They have also considered the influence of the amount of isotopically labelled amino acid to the total dietary intake administered in some studies, and the feeding regimens used. This has led them to recalculate a number of Young's results, and conclude that many tend to overestimate essential amino acid requirements.

Table 1.5 Routes of obligatory amino acid loss other than oxidation (after Fuller and Garlick, 1994)

ROUTE OF LOSS	*ESTIMATED % LOSS/d*
Losses from the skin	>5%
Losses via the GI tract	15-30%
Urinary Excretion	>5%
Irreversible modification	About 5%
Synthesis of non-protein substances	>5%

With respect to the inflow of *de novo* amino acids, there is accumulating evidence that essential amino acids synthesised through the actions of the colonic microflora may be available to the body in functionally significant amounts. Millward and colleagues have discussed the implications of *de novo* synthesis of essential amino acids in normal adults in the context of requirements. *'The implications of...de novo synthesis of indispensable amino acids are profound... This makes the concept of protein quality meaningless with the potential for qualitative modification of the amino acid balance by the lower gut...'* (Millward *et al*, 1991)

In summary, ^{13}C tracer balance methods have been used to determine essential amino acid requirements in adults, resulting in new proposals for intakes which are up to 2 to 3 times higher than those currently recommended. However, the interpretation of the results which led to these proposals has come under criticism, recently summarised (Waterlow, 1996). Jackson (1993) has also pointed out that ^{13}C-labelled tracers can only provide data on the fate of carbon through the body, and so cannot be directly used to determine amino acid *nitrogen* requirements. The important suggestion that *de novo* synthesised essential amino acids may be available to the body in functionally significant amounts is not taken into account within the theoretical framework of ^{13}C tracer studies. Stable isotope methodologies employing ^{15}N-labelled urea, which trace the metabolic fate of urea-nitrogen in the body, represent a further approach to study protein, amino acid and nitrogen requirements in humans. Before discussing the contribution of some of these studies, it is first important to consider urea metabolism in general.

1.7 Urea metabolism: a classical view

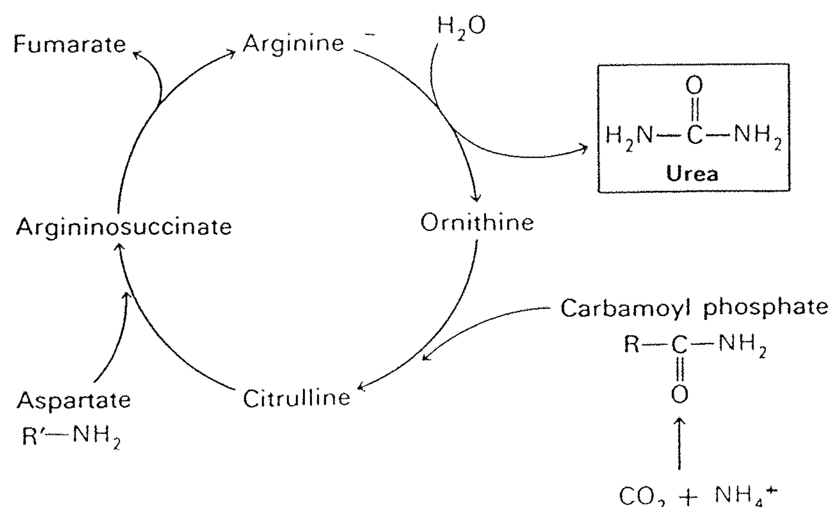
Amino acids in excess of those needed for the synthesis of proteins and other biomolecules cannot be stored in the body or excreted directly. Instead, most of the amino groups of surplus amino acids are used to form urea, while the carbon skeleton is used as metabolic fuel. Urea is primarily excreted by the kidneys into the urine and accounts for about 90 % of urinary nitrogen excretion and about 80 % of body nitrogen excretion.

Rouelle was the first to identify urea in urine in 1773, and it was soon realised that this was the major nitrogen containing excretion product in humans and other mammals (Carpenter, 1994). In 1823, Prevost and Dumas demonstrated that the kidneys were not the site of urea synthesis after urea accumulated in the blood of rabbits and cats when the kidneys were removed. Shroeder showed that urea was formed when the liver was perfused with ammonium salts in 1882, but it was not confirmed that the liver was the sole site of ureagenesis until 1924, when Bollman observed that urea synthesis was completely

suppressed with the removal of the liver (Munro, 1964) In 1932, Krebs and Henseleit proposed the arginine-ornithine cycle (or urea cycle) as the mechanism for urea synthesis, which was the first cyclical metabolic pathway ever described (Figure 1.2). The understanding of the mechanism by which amino groups become available for urea synthesis was helped with the discovery of the transaminases by Braunstein and Kritzman in 1937 (Munro, 1964).

In the formation of urea, the amino group of an amino acid is transferred to α-ketoglutarate, forming glutamate. Glutamate is then oxidatively deaminated to yield ammonium ions. The transfer of an amino group from an amino acid to an α-keto acid is known as *transamination.* During degradation, most amino acids need to transfer their amino group to α-ketoglutarate, whereas serine and threonine can be directly *deaminated* to form ammonium ions. High levels of ammonium ions are toxic to humans and may lead directly to brain damage. Some of the ammonium ions released from amino acid degradation can be channelled into the biosynthesis of nitrogenous molecules, but most are used to form urea and then excreted.

Figure 1.2 The urea cycle



Classically, it was assumed that urea was an inert metabolite of protein metabolism, and that the production rate in the liver was equal to the excretion rate in the urine. However, research over the past 50 years has conflicted with

this view. When ^{14}C-labelled urea was administered to mice and cats (Leifer *et al*, 1948; Kornberg and Davies, 1952), label was subsequent recovered as $^{14}CO_2$ on the breath, indicating that urea was being continually broken down in the body. It was Walser and Bodenlos (1959) who first demonstrated this phenomenon in humans, and this opened the way for the development of techniques to study urea kinetics.

1.8 The study of urea kinetics

Walser and Bodenlos (1959) gave of a single intravenous dose of urea labelled with two ^{15}N atoms - [$^{15}N^{15}N$]urea - to healthy individual consuming their habitual diet. By following the excretion of label into the urine and plasma, they concluded that only 80 % of urea continually synthesised on average was excreted, with the remaining 20 % undergoing hydrolysis. The hydrolysis of urea was presumed to occur through the actions of bacterial urease, since urea hydrolysis was almost abolished after the administration of a broad-spectrum antibiotic. It is now known that in normal individuals, over 99.9 % of gastrointestinal bacteria reside in the colon (Gustafsson, 1982).

The technique employed by Walser and Bodenlos to study urea kinetics was novel in that the use of [$^{15}N^{15}N$]urea allowed a distinction between urea that had passed through the body unchanged, and that which had been hydrolysed in the colon and then subsequently retained or reformed into urea, information which could not be determined from the study of urea kinetics using [^{13}C]urea (Jones *et al*, 1969; Long *et al*, 1978). In their analysis, urinary urea was reacted with sodium hypobromite to form nitrogen gas which was then analysed by mass spectrometry. During this reaction, Walser and colleagues (1954) had demonstrated that the nitrogen gas formed derives both atoms from a single urea molecule. Hence, [$^{15}N^{15}N$]urea forms [$^{15}N^{15}N$] gas when reacted with sodium hypobromite, and the dilution of [$^{15}N^{15}N$]urea in the urine was used to calculate the rate of endogenous production of urea. Some of the administered label was not excreted directly, but appeared in the urine as [$^{14}N^{15}N$]urea. This represents labelled urea which is hydrolysed in the colon to form

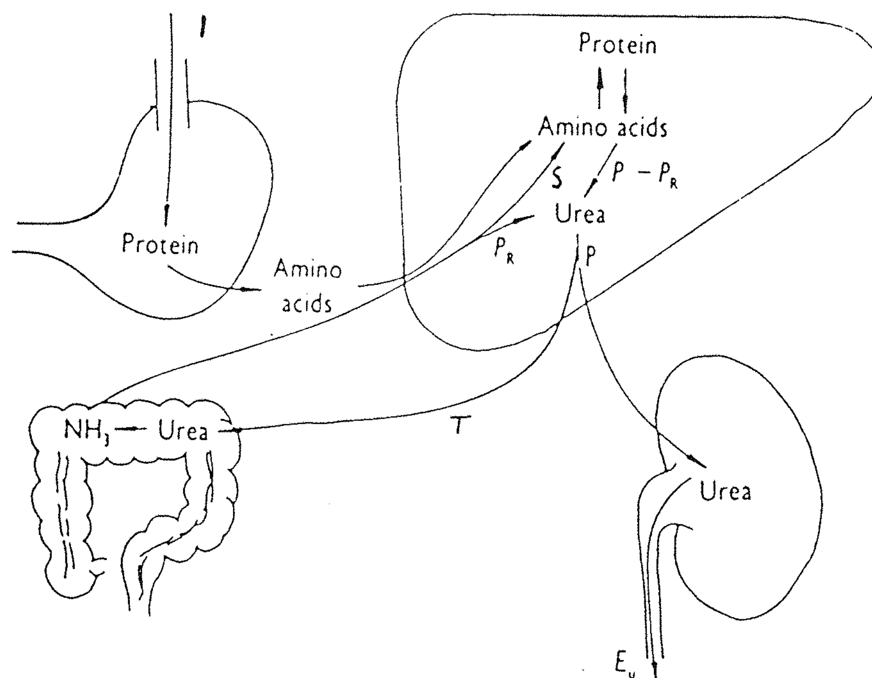
^{15}N-ammonium ions, which are then reabsorbed by the body and used to form urea. Because the vast majority of nitrogen in the body is present as ^{14}N, this urea will be formed and excreted as [^{14}N^{15}N]urea. The appearance of [^{14}N^{15}N]urea in urine can then be used to calculate the extent to which endogenous urea is reformed to urea after hydrolysis. The remaining label not excreted in the urine is assumed to be accreted in the body nitrogen pool, presumably in some metabolically useful form, and represents endogenous urea which has been *salvaged* and retained by the body. A model of the normal metabolic pathways for urea is shown in figure 1.3.

Two general non-invasive approaches to study urea kinetics using [^{15}N^{15}N]urea have subsequently been developed. The *single dose* method, first used by Walser and Bodenlos, traces the excretion of a single oral bolus of [^{15}N^{15}N]urea into the urine over a set period of time. The single dose method has the advantage of being simple to apply and so can be used to study urea kinetics in larger groups of individuals under free-living conditions, but the mathematical model which is employed is complex since it relies on the interpretation of a isotopic decay curve and the estimation of the size of the urea pool. A single dose method has recently been developed which measures cumulative urinary excretion of isotope over 48 h and is more easy to interpret (Jackson *et al*, 1993). This has been applied to the study of urea kinetics in pregnant and non-pregnant women (McClelland *et al*, 1992; McClelland *et al*, 1993), female vegetarians on their habitual diet (Bundy *et al*, 1993) and Chilean children on animal or vegetable rich diets (Bickerton *et al*, 1996).

The *intermittent dose* method was developed by Picou and Phillips to study urea kinetics in malnourished and recovered children on high and low protein diets, and allowed the application of steady state kinetics to a stochastic model. They administered smaller oral doses of [^{15}N^{15}N]urea to subjects at intervals, rather than as a single dose. Once a steady isotopic state had been achieved, the ratio of labelled [^{15}N^{15}N] and [^{14}N^{15}N]urea in the urine to unlabelled urea was used to calculate urea production, excretion and hydrolysis (1972). Although the method is more labour intensive to apply, it allows the application of a simpler mathematical model to study urea kinetics. Wolfe (1981) developed the model

further by giving a priming dose of [$^{15}N^{15}N$]urea. This meant that isotopic equilibrium was achieved more rapidly in the urea pool and therefore urea kinetics could be calculated over a shorter study period. Wolfe also gave label intravenously to rule out any possible hydrolysis of [$^{15}N^{15}N$]urea in the upper GI tract. *Helicobacter pylori*, a spiral gram-negative bacteria which can infect the upper intestinal tract, contains an active urease and may hydrolyse isotopically labelled urea before it is presented to the colon, leading to spurious results for urea hydrolysis. However, intravenous presentation of dose is invasive, and therefore not suitable for many studies.

Figure 1.3 Diagram of the normal metabolic pathways for urea (after Langran *et al*, 1992)



where I = urea intake, P = total endogenous urea production, Eu = urinary urea, T = hydrolysed urea, Pr = urea formed from salvaged nitrogen and S = urea nitrogen retained in the body

Jackson *et al* (1984) have compared the oral, intravenous and intragastric presentation of isotopically labelled urea, and concluded that the route of isotope administration had no effect on the measurement of urea kinetics. They developed a two-pool model for the measurement of urea kinetics in man using a non-invasive primed/intermittent oral dose of [$^{15}N^{15}N$]urea. Since then there

has been critical examination of the models used to study urea kinetics (Matthews and Downey, 1984; Jahoor and Wolfe, 1987; Hibbert and Jackson, 1991; Hibbert *et al*, 1992), and it has been concluded that the non-invasive primed/intermittent oral method is reliable, has good repeatability, and is suitable for patient study with more accuracy than single dose methods.

It should be noted that most studies of urea kinetics rely on the assumption that administered [$^{15}N^{15}N$]urea not excreted in the urine passes to the colon where it is hydrolysed. The extent to which this assumption is correct is not clear. In order to quantify the fate of urea-nitrogen released through bacterial hydrolysis, labelled urea should ideally be presented directly to the site of hydrolysis, the colon. However, this is difficult to apply in practise. Before these considerations are discussed further, it is important to look at the contribution of studies of urea kinetics, and how these have influenced the understanding of nitrogen metabolism and essential amino acid requirements in adults. First, the role of the colon in urea hydrolysis will be considered.

1.9 Urea hydrolysis and the colon

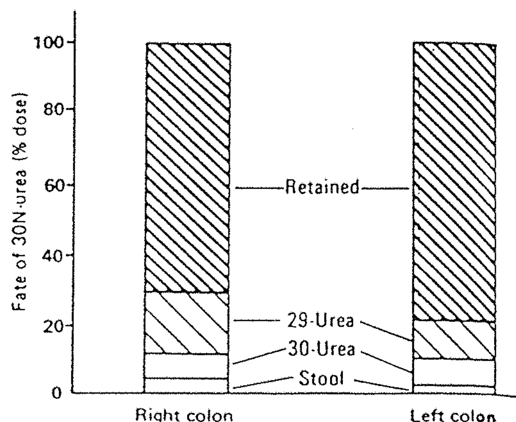
The large intestine has traditionally been considered as a useful but dispensable organ for the conservation of salt and water (Moran, 1992). Much work has be carried out on the dysfunctional colon, and the results have been extrapolated to explain the role of the normal colon. In a recent review, Moran (1992) has divided the function of the normal colon into three areas: a *capacity function* for the absorption of water and electrolytes, and for the controlled evacuation of stool, a *reservoir function* for bacterial metabolism, and a *metabolic function* which has effects on whole-body metabolism, including nitrogen metabolism.

Walser and Bodenlos (1959) indicated the role of the gut bacteria in urea hydrolysis after they had observed that hydrolysis was almost completely abolished after subjects were given a wide-spectrum antibiotic. It had previously been demonstrated that the concentration of ammonium ions in the blood

draining from the colon is higher than in any other part of the bowel, and ten-times higher than that in the inferior vena cava (Silen *et al*, 1955). Summerskill and Wolpert (1970) estimated that 75 % of the ammonium ion production in the gastrointestinal tract was through the actions of the colonic bacteria, and concluded that a major source of this was from the hydrolysis of endogenous urea. This idea was echoed by Wrong *et al* (1970) who observed no excretion of urea in the stool of normal adults, suggesting that urea entering the colon was completely degraded. They also confirmed that urea degradation was almost entirely a function of the colonic bacteria, since administering a mixture of antibiotics to the same subjects resulted in faecal urea concentrations similar to plasma urea levels.

Only approximately 10 % of the urea needed to account for the production of ammonium ions observed enters the colon through the ileocaecal valve, and so it was suggested that urea must pass into the colon in substantial quantities from the circulation (Gibson *et al,* 1976). The permeability of the colon to urea had been disputed by some investigators (Levitan and Billich, 1968; Wolpert *et al*, 1971) who reported little movement of urea from the circulation into the colonic lumen, and *vice versa*. Moran and Jackson (1990*b*) resolved this controversy when they demonstrated the rapid appearance of [$^{15}N^{15}N$]urea in the urine after a single dose had been placed directly into the colon of patients with a loop colostomy, indicating that the colon is permeable to the intact urea molecule. They also recovered a greater proportion of the dose in the urine as [$^{15}N^{15}N$]urea when label had been placed in the left defunctioned colon of patients (29%) compared with the left or right functioning colon (9 and 4 % respectively). This was thought to be related to the decreased bacterial activity in the defunctioned colon. Extending their studies to normal adults by the use of a colonoscope, they observed that 6 and 4 % of a dose of [$^{15}N^{15}N$]urea placed directly into the right and left colon respectively was recovered intact in the urine (1990*a*). Recovery of dose as urinary [$^{14}N^{15}N$]urea was 18 and 13 % respectively, and less than 4 % of the dose appeared in the stool, suggesting that the majority of the label had been retained in the body (Figure 1.4).

Figure 1.4 The fate of [$^{15}N^{15}N$]urea instilled into the colon after 72 hours (after Moran and Jackson, 1990*a*)



The idea that urea may traverse cell and tissue membranes by passive lipid-phase diffusion has recently been challenged with the characterisation of a human urea transporter, HUT11, in erythroid and renal tissues (Olives *et al*, 1994). You and colleagues (1993) have identified similar urea transporters in rabbit tissues, including the colon, and suggest that these may be regulated through the actions of vasopressin. Both these reports indicate that the transport of urea in the body is a controlled process, and this may be of importance in determining how the body can adapt to changes in dietary protein intake in which the hydrolysis of urea seems to plays an important role.

1.10 The functional significance of urea salvage

Hydrolysis of urea, and the subsequent salvage of the nitrogenous part, is an integral part of the body's mechanism to maintain nitrogen equilibrium in situations of nitrogen economy. In simple terms, urea salvage increases as the *intake of nitrogen falls when the demand is fixed*, or when the *demand for nitrogen increases when the intake is fixed* (Jackson and Wootton, 1990).

Growth is a very important physiological condition when the demand for nitrogen increases. *In utero*, there is a need for the deposition of both maternal and

foetal tissue throughout the pregnancy. It has been shown that urea salvage was higher in trimester one of pregnant women compared with non-pregnant controls, even though dietary protein intake was higher in the pregnant women, indicating an increased maternal demand during this period (Forrester *et al*, 1994). McClelland *et al* (1996) have reported that on average, over 45 % of urea production was salvaged in healthy women during pregnancy. In the new-born child, it has been observed that about 75 to 80 % of urea production was salvaged in the colon, with the majority of this retained in the body (Wheeler *et al*, 1991; Steinbrecher *et al*, 1996). In children studied during catch-up growth, following malnutrition or disease, Jackson and colleagues observed that urea salvage on a low protein diet was 61 % of production, compared with 39 % on a higher protein diet. Aside from growth, demand for nitrogen may also increase due to a pathological insult, and it has been shown that urea salvage is increased following major surgery (Jackson and Moran, 1992) and in individuals with homozygous sickle cell disease (Jackson *et al*, 1988).

It is also important to consider changes in urea metabolism which occur when the intake of nitrogen falls, *i.e.* when dietary supply of protein is limiting, either habitually or experimentally. It has been understood for at least a century that the body is able to accommodate a reduced protein intake in order to maintain body nitrogen balance. This has been generally considered as an adaptive response which is beneficial to the individual. Adaptations can be considered at three distinct levels: *genetic*, *physiological*, and *social*. *Genetic* adaptations may be viewed classically as Darwinian, in that the genotype of the organism is altered and the adaptation is heritable. Consequently, the genetic make-up of an individual will determine the extent to which non-heritable *physiological* adaptations are possible. Finally, the capacity for physiological adaptation will influence any *social* adaptations which are necessary (Waterlow, 1985).

Waterlow (1985) has defined the processes which occur to maintain nitrogen homeostasis in situations of nitrogen economy as a *physiological* adaptation, the purpose of which is to improve the efficiency of amino acid utilisation. This adaptive response is reversible, and allows a new metabolic steady-state to be achieved. Although adaptation confers some benefit to the individual, there is

usually an associated metabolic cost. Hence, some physiological processes may be sacrificed or down-regulated in order that others be preserved. For example, the rate of synthesis and degradation of protein (called *protein turnover*) in peripheral tissues is reduced, in order that nitrogen is channelled to vital organs such as the liver. The cost to maintain a nitrogen supply to the liver in this situation, therefore, is a lowered capacity for peripheral tissues to respond to any internal or external change. In rats, it has been observed that physiological adaptation to a lowered protein intake also involves a decrease in the rate of urea production, and in urea excretion rate. In the short-term, there is a reduction in the *activity* of the five urea cycle enzymes, whereas over the long-term, the *amount* of enzyme may be reduced (Schimke, 1962). Enzymes involved in the degradation of amino acids can also be regulated (Harper, 1968). Both these effects will contribute to nitrogen economy by causing a reduction in urea production. The reduction in the rate of urea excretion when dietary protein is limiting makes more urea available for bacterial hydrolysis, salvage, and retention in the body nitrogen pool. Studies which have observed this phenomenon can be considered into two sections; those where protein intake is lowered experimentally, and those where protein intake is habitually low.

1.10.1 Urea kinetics when experimental diet is limiting in protein

Urea salvage has been investigated in patients with chronic renal failure. These *uraemic* patients have elevated plasma urea levels, disturbances in hydrogen ion concentration, and abnormalities in water and electrolyte balance. Part of their clinical management is the consumption of a low protein diet, and the possibility that they may utilise salvaged urea for protein synthesis is of great interest. Giordano and colleagues (1968) observed that uraemic patients consuming a low protein diet and administered with ^{15}N-labelled urea incorporated three times more label into non-essential amino acids than a control subject. Administration of antibiotics greatly reduced urea utilisation, again confirming the role of the colonic bacteria. It was subsequently reported that uraemic patients utilised over 10 times more urea nitrogen for albumin synthesis compared with normal subjects, and that the efficiency of utilisation

was higher in uraemic patients when consuming a 30 g protein/d diet rather than one providing 70 g/d (Varcoe *et al*, 1975).

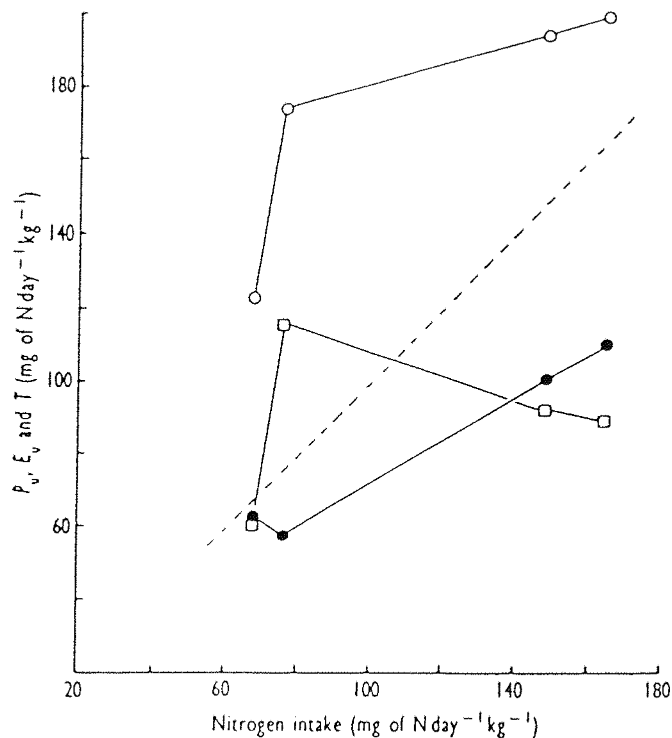
Urea kinetics have also been studied in malnourished and recovered children consuming high and low protein diets (Picou and Phillips, 1972). It was reported that on moving from a high to a low protein, urea salvage increased from 32 to 69 % of production in the recovered children and from 38 to 61 % of production in the malnourished children, and that the proportion of salvaged urea retained in the body increased in both groups. This suggested that the changes in urea metabolism were in an attempt to conserve nitrogen in an adaptive response to a low nitrogen intake. Jackson *et al* (1990) have studied urea kinetics in young children recovering from severe malnutrition. Urea salvage on a high protein diet was 39 % of production, compared with 61 % on a low protein diet, and it was calculated that the increase in urea salvage on the low protein diet was equivalent in magnitude to the decrease in dietary protein intake. Therefore, the 'available nitrogen' from the diet plus salvage was similar to that on the high protein diet. They also reported that different proportions of fat or carbohydrate in the diet modified urea kinetics, but this was not as important as the *quantity* of protein ingested.

Two recent studies of urea kinetics in normal individuals consuming different quantities of protein have been conducted. Langran and colleagues (1992) observed that 46 % of urea production was salvaged in five adult males consuming 70 g protein/d, with 90 % of the salvaged urea nitrogen retained in the body. Measured urea production rate was not significantly different in the same subjects consuming a 35 g protein/d diet, but urea salvage increased to 64 % production. It had been reported previously (Stephen, 1980) that increasing dietary intake of non-starch polysaccharides (NSP) increased the bacterial mass of the stool by promoting growth of the colonic microflora. Langran and colleagues noted that the addition of NSP to subjects consuming the low protein diet significantly increased the bulk and weight of stool, but had no significant effect on urea kinetics. They concluded that adaptation to a low protein diet was not associated with a large drop in urea production but with an increase in urea salvaging. As with the study in children (Jackson *et al*, 1990),

the decrease in nitrogen intake matched the magnitude of colonic urea salvage, such that the intake plus salvage, the body's 'available nitrogen', remained the same. Subsequently, Danielsen and Jackson (1992) investigated the limit of metabolic adaptation to a lowered dietary protein intake. Six healthy adult males were fed diets containing either 74 g or 30 g protein/d, and urea kinetics were measured on the fourth day. On the higher protein diet, 40 % of urea production was salvaged with 69 % of that being retained in the body. On the lower protein diet, nitrogen balance was not maintained, and there was a significant fall in urea production, with only 46 % of this salvaged. Hence, urea salvage in subjects consuming a diet of 30 g protein/d was only half that of previous individuals consuming 35 g protein/d, as studied by Langran *et al* (1992). These results suggest that the *minimum dietary protein requirement* which will allow the maintenance of nitrogen equilibrium in normal adults through the salvage of endogenous urea nitrogen is between 30 and 35 g protein/day (Figure 1.5).

Exogenous urea has been used to supplement diets which are low in total nitrogen and improve nitrogen balance, as discussed previously in section 1.4. Recently, Meakins and Jackson (1996) studied urea kinetics in six healthy men consuming four different diets: a 70 g protein/d reference diet (REF), a low protein diet supplying 30 g/d (LP), the same low protein diet supplemented with 6.9 g of urea (LP-U1), and the low-protein diet supplemented with 13.7 g urea (LP-U2). The REF and LP-U2 diets supplied the same amount of *total* nitrogen to subjects. Apparent nitrogen balance was better in subjects consuming LP-U2 compared with LP and LP-U1, and was not different to apparent nitrogen balance in subjects consuming the REF diet. All of the low-protein diets caused a 40 % decrease in urea production compared to the REF diet, but urea salvage was increased with the highest level of urea supplementation, with over 80 % retained by the body.

Figure 1.5 Comparison of urea kinetics on different protein intakes (after Danielsen and Jackson, 1992)



Open circle = urea production, closed circle = urinary urea excretion, open box = urea hydrolysis. The dotted line indicates the equivalent value of nitrogen intake on both axes.

1.10.2 Urea kinetics when habitual diet is limiting in protein

Several Japanese workers have studied urea kinetics in groups of Papua New Guinea (PNG) Highlanders, a people whose diet has been classically viewed as too low in nitrogen to be sustaining, even though the Highlanders appear to enjoy good health. Tanaka and colleagues (1980) observed that PNG adults and children retained more ^{15}N in the body when consuming a low protein diet than Japanese controls, after a single oral dose of ^{15}N-labelled urea. ^{15}N enrichment in plasma protein was also higher in PNG subjects and controls consuming a low protein diet, than in controls consuming a standard protein diet. It was subsequently demonstrated that PNG Highlanders incorporated more ^{15}N into serum protein than controls, even when consuming a diet adequate in protein (1.34 g/kg/d), suggesting that endogenous urea nitrogen was used for the deposition of body tissue (Miyoshi *et al*, 1986). Rikamaru and

colleagues (1985) reported that serum protein levels of PNG Highlander adults were in the normal range even though average daily protein intake was low (32.2 ± 8.6 g/d). Incorporation of an oral dose of ^{15}N-labelled urea into serum protein was negatively correlated with urinary nitrogen excretion, indicating that PNG Highlanders in a poorer state of nutrition utilised more urea nitrogen for the synthesis of serum protein. These studies suggest that PNG Highlanders are able to efficiently utilise salvaged urea nitrogen, and that this is an essential adaptation to their habitual low protein diet. Additionally, Bundy *et al* (1993) have studied urea kinetics in six female vegetarians consuming their habitual diet and demonstrated that salvage of urea nitrogen was increased as individual protein intake fell.

In summary, these studies of urea kinetics have demonstrated that the body is able to adapt to a lowered protein intake through an increase in the hydrolysis, salvage, and retention of urea nitrogen, in an attempt to maintain nitrogen equilibrium. The limit of this adaptive mechanism is apparently reached when protein intake falls below 35 g/d in normal adult males. The form in which salvaged urea nitrogen is retained in the body has been a matter of controversy. With specific regard to the thesis, there has been increasing evidence to suggest that essential amino acids formed *de novo* by the colonic bacteria may be utilised by the host, although this idea has generally been regarded with scepticism .

1.11 Evidence for utilisation of essential amino acids formed *de novo* in the colon

When Walser and Bodenlos first demonstrated the continual hydrolysis of urea in normal adults, they thought that the phenomenon held no physiological significance. Walser later suggested that it was purely a futile cycle which represented an isotopic exchange phenomenon (Jackson *et al*, 1982). Ammonia released from urea hydrolysis is either reformed to urea in the liver, or transaminates with amino acids in the portal tract, with no net accretion of nitrogen. However, the correlation between the intensity of urea hydrolysis and

the physiological state of the individual, as discussed in the previous section, suggests that salvage does play a role in maintaining nitrogen equilibrium in situations of nitrogen economy.

Originally, it was suggested that the body utilises hydrolysed urea by fixing the salvaged nitrogen, in the form of ammonium ions, into amino acids. The routes of fixation are limited to amination of α-ketoglutarate to form glutamine, and accretion of ammonium ions through the serine/glycine interchange pathway. Further transamination reactions may then incorporate the salvaged nitrogen into other amino acids. However, it is unclear whether the body has the capacity to fix such potentially large amounts of salvaged ammonium ions through these metabolic routes. Even if this were so, the result may be an imbalance in free amino acid concentrations which would lead to an increase in amino acid degradation. This could again result in no net accretion of salvaged nitrogen.

A further suggestion was that amino acids were synthesised *de novo* by the colonic bacteria, and were subsequently available to the body. It is well established in ruminant nutrition that the microflora of the fore-gut play a critical role in the conversion of simple nitrogen sources, such as urea, to amino acids. These can then be utilised by the host (Briggs, 1967). Giordano and colleagues (1968) were the first to demonstrate ^{15}N enrichment in the lysine fraction of plasma albumin in one normal person and patients with chronic renal failure consuming normal protein, low protein and amino acid diets, and given intermittent oral doses of ^{15}N-labelled urea. Isotopic enrichment was higher in the patients compared to the normal subject, up to 0.08 APE on the low protein diet. Lysine is one of only two amino acids which can neither be synthesised nor aminated in the body. Therefore, ^{15}N enrichment in lysine after administration of ^{15}N-labelled urea is indicative of *de novo* lysine formation in the colon. It was also observed that label from administered ^{14}C-glucose was incorporated into essential amino acids isolated from albumin, and overlapped with the ^{15}N labelling, suggesting *de novo* formation of essential amino acids. Sheng *et al* (1977) observed labelling in the imidazole ring of histidine in subjects given ^{15}N-ammonium chloride, and the high enrichment of ^{15}N in faecal bacteria suggested the colon as a possible site of histidine synthesis. Tanaka and

colleagues (1980) noted that PNG Highlander adults and children, plus a Japanese control group consuming a low protein diet, all showed incorporation of ^{15}N into the lysine fraction of total plasma proteins in the range 0.01 to 0.05 APE after a single oral dose of ^{15}N-labelled urea. She has also demonstrated (1982) that ^{15}N label from urea was incorporated into essential amino acids, including lysine, in rats, with this effect being increased on the consumption of a low protein diet and abolished after treatment with antibiotics. More recent work by Torrallardona and colleagues (1993*a,b*) has demonstrated the incorporation of ^{15}N and ^{14}C into lysine in the pig and rat when the only dietary source of nitrogen provided was ammonium chloride. This effect was not seen in gnotobiotic rats.

Heine and colleagues (1982) have investigated the functional significance of the relatively high urea content of human breast milk and reported that ^{15}N-labelled urea was incorporated into the bacterial fraction of infants receiving both mother's milk and cow's milk. They noted that the rate of incorporation was 30 times higher in an infant suffering from kidney insufficiency, and that *Bifidobacteria infantis* isolated from faeces of the breast fed infants directly utilised urea in culture medium. They have gone on to demonstrate the direct utilisation of ^{15}N labelled yeast protein instilled directly into the colon of infants with colostomies and shown that between 87.1 and 98.1% of the administered dose was absorbed, with between 79.0 and 94.2 % of the dose being retained in the body pool respectively (Heine *et al*, 1987). In a further study, an average of 85.5 % of a single dose of ^{15}N-labelled bifidobacteria instilled into the colon of infants with colostomies was absorbed (Heine *et al*, 1991).

1.12 The current debate

The metabolic significance of urea nitrogen salvage in normal individuals has remained a contentious issue ever since it was first identified. The intensity of urea salvage appears to be a controlled process. Although the mechanisms of this control are not fully understood, it is clear that as dietary protein intake falls, there is a proportional increase in urea hydrolysis and salvage to compensate

for the lowered intake, while the rate of urea production remains relatively constant (Langran *et al,* 1992). The salvage mechanism would therefore appear to be an integral part of the body's capacity to maintain nitrogen homeostasis over a range of protein intakes.

The accumulating evidence that the body may utilise essential amino acids synthesised *de novo* through the actions of the colonic bacteria is of fundamental importance to the current debate concerning adult essential amino acid requirements. It has potential to help explain the large discrepancy in recommendations as calculated using studies of nitrogen balance, which include this possibility within the theoretical model, and recent ^{13}C tracer balance studies, which do not. It may also explain why the rate of urea hydrolysis and retention of nitrogen is so high under certain physiological conditions, even though the metabolic routes to fix ammonium ions appear so limited. One of the more outstanding questions surrounding the debate is whether the supply of *de novo* amino acids has any functional significance in normal adults. In order to address this question, it is vital to accurately *quantify* the salvage and retention of urea nitrogen from the colon. Urea kinetics methodology assumes that the difference between urea production and excretion rates represents urea which enters the colon and undergoes hydrolysis. However, there are other possible routes of loss which may be of importance when trying to quantify salvage. Urea is excreted in the sweat, and the extent of this is difficult to quantify experimentally. It has also been demonstrated that there is a diurnal fluctuation in the size of the urea pool, as determined by fluctuations in plasma urea concentration (Price *et al*, 1994). This is important to note, since the theoretical model of urea kinetics assumes that the size of the urea pool remains constant over the period of measurement. Therefore, any changes in the urea pool size during measurements will be interpreted incorrectly, and will result in an incorrect value for the rate of urea hydrolysis. Additionally, the presence of bacterial urease in the oral cavity has been demonstrated, which would lead to hydrolysis of an orally administered dose of ^{15}N-labelled urea and lead to spurious results for colonic hydrolysis. In summary, it is uncertain whether measurements of urea kinetics can be used to accurately quantify the hydrolysis, salvage, and retention of urea nitrogen in normal adults. Instilling

^{15}N-labelled urea directly at the site of hydrolysis is one way to eliminate the limitations imposed by conventional urea kinetics methodology. This has been achieved in adults by Moran and Jackson using a colonoscope, but this invasive method has obvious limitations to larger groups of normal individuals.

Isotopically labelled lactose-ureide, a glycosyl ureide formed from the covalent attachment of urea to lactose, has recently been used as a non-invasive marker of orocecal transit time in a single adult (Heine *et al*, 1995). Lactose-ureide is reported to be resistant to digestion in the upper gastrointestinal tract but may be hydrolysed by certain species of colonic bacteria, releasing the urea part for further hydrolysis. Orally administered lactose-ureide therefore offers a potential means of transporting a known dose of isotopically labelled urea directly to the colon, and subsequently a means of *non-invasively quantifying* the salvage of urea nitrogen in normal adults.

1.13 Aims and objectives of the thesis

The aim of the thesis is to develop a novel method to non-invasively quantify the fate of urea nitrogen salvaged in the colon of normal adults consuming a diet marginally adequate in protein. It is hoped that this will provide a new tool to help more fully understand the functional significance of urea salvage, and the current debate surrounding protein, amino acid, and nitrogen requirements.

Orally administered lactose-ureide provides a potential means to non-invasively deliver a known dose of isotopically labelled urea to the colon of normal individuals. By following the excretion of label into the urine and stool, it may be possible to quantify the hydrolysis, salvage, and retention of urea.

The approach taken can be divided into three parts:

1. *Synthesis and analysis of the purity of two isotopically labelled lactose-ureides.* This will involve employing a method to synthesise both lactose [^{13}C]ureide and lactose [^{15}N^{15}N]ureide from lactose and labelled urea, and a variety of analytical techniques to test the integrity and purity of the two labelled lactose-ureides.

2. *Observe the metabolism of lactose [^{13}C]ureide in normal adults.* This will involve setting up a clinical trial and orally administering lactose [^{13}C]ureide to adult subjects consuming a diet marginally adequate in protein. By following the excretion of label on breath, the suitability of lactose-ureide to non-invasively deliver isotope directly to the colon can be assessed.

3. *Observe the metabolism of lactose [^{15}N^{15}N]ureide in normal adults.* This will involve setting up a clinical trial and orally administering lactose[^{15}N^{15}N]ureide to adult subjects consuming a diet marginally adequate in protein. By following the excretion of label into urine and stool, it is hoped that urea hydrolysis, salvage and retention may be quantified.

CHAPTER 2

Materials and Methods

2.1 General Introduction

The methods chapter is presented in four sections. The first describes the techniques used in the synthesis and analysis of lactose-ureide isotopically labelled with either ^{13}C or ^{15}N. In the second, a clinical trial is described, including some subject data and details of the diet that was administered. General methods used to analyse samples generated throughout the clinical trial are detailed in section three, and the last section contains theoretical considerations. Unless otherwise stated, all reagents were of analytical grade and were obtained either from the Sigma Chemical Company Limited, Poole, Dorset, England or BDH-Merck, Poole, Dorset, England. All figures referred to in the text are shown in appendix 2A.

2.2 Synthesis and analysis of lactose-ureides

Lactose ureide is a glycosyl-ureide which is formed when urea covalently attaches to lactose under acidic conditions. The molecular structure, along with the proposed sites of enzymatic cleavage, is shown in figure 2.1. A pilot synthesis of lactose-ureide using unlabelled urea was carried out initially, followed by the synthesis of labelled lactose [^{13}C]ureide and lactose [^{15}N^{15}N]ureide. A range of techniques were employed to assess the integrity, purity, and molecular weight of lactose-ureides.

2.2.1 Synthesis

Samples of lactose-ureide were synthesised according to the method of Hofmann (1932). For the pilot synthesis, 20 g lactose were dissolved in 40 ml hot water in a round bottom flask, and cooled to 50 °C. 10 g urea and 2 ml 200g/l sulphuric acid solution were added. The solution was mixed, covered with parafilm, and placed in a waterbath at 42 °C for 12 days. The volume of liquid was then reduced to about half by rotary evaporation, and the solution was refrigerated at 4 °C. Crystals formed over 4 days. These were filtered and re-dissolved in the minimum volume of hot water and ethanol (1:1 v/v). This solution was refrigerated for three days, and the resulting crystals were then filtered from the solution and desiccated. Some of these crystals were used to seed the remaining liquor, which was refrigerated for a further three days. After weighing the dried crystals, the total yield was calculated to be 48 % of the theoretical maximum. Lactose [^{13}C]ureide and lactose [^{15}N^{15}N]ureide were subsequently synthesised using [^{13}C]urea and [^{15}N^{15}N]urea respectively (>99 APE; Cambridge Isotope Laboratories, Cambridge, MA, USA). The same procedure was followed, except that the starting quantities of substrate were one fifth of those used for the pilot synthesis. The yields of lactose [^{13}C]ureide and lactose [^{15}N^{15}N]ureide were calculated to be 50 and 46 % of the theoretical maximum respectively.

2.2.2 Analysis of molecular integrity

It was important to analyse samples of lactose-ureide to confirm that the synthetic procedure had been successful. Two absorption-spectroscopy techniques were employed to identify specific chemical similarities and differences between pure lactose and unlabelled lactose-ureide. Additionally, two mass-spectrometry analyses were performed to confirm the molecular weight of lactose [^{13}C]ureide and lactose [^{15}N^{15}N]ureide.

2.2.2.1 Infra-red absorption-spectroscopy (IR)

The theory underlying techniques of absorption-spectroscopy is that atoms, and the chemical bonds between atoms, absorb electromagnetic radiation at specific wavelengths. The detection of this absorption allows a qualitative identification of certain chemical species in an unknown molecule, with reference to known absorption spectra. IR techniques can measure absorption in the range of 12,500 to 10 cm^{-1}, and are more specific than UV or visible light absorptions. Mulls of both lactose and unlabelled lactose-ureide were prepared by dissolving a small sample of each (1-2 mg) in liquid paraffin. These were separately transferred to a clean sodium chloride block and analysed for absorption between of 4000 and 200 cm^{-1} (Pye Unicam Infrared Spectrophotometer, Phillips). Representative print-outs of the absorption spectra for lactose and unlabelled lactose-ureide are shown (Figures 2.2 and 2.3). Liquid paraffin absorbs IR radiation between about 2800 and 3000 cm^{-1}, and about 1300 and 1500 cm^{-1}. Absorption in the range of about 3100 and 3500 cm^{-1} in both graphs corresponds to the presence of OH species in lactose and lactose-ureide. The area marked (*) in figure 2.3 for lactose-ureide is absent in figure 2.2, and is taken to represent absorption of the peptide bond between lactose and urea (1660 cm^{-1}) and the C-N and C=O bonds of urea (1620 cm^{-1}).

2.2.2.2 Nuclear magnetic resonance (NMR)

NMR is a spectroscopic technique which employs electromagnetic radiation of frequencies near the middle of what is called the radiofrequency part of the

spectrum. NMR is restricted to identifying chemical species which posses a magnetic charge, but it is a very sensitive technique and allows excellent resolution of absorption. About 5 to 10 mg of lactose and unlabelled lactose-ureide crystals were dissolved separately in 0.5 ml deuterated dimethyl sulphoxide. Samples were then analysed (Hitatchi-FT-NMR-R-1500 Spectrometer). Representative print-outs of the absorption spectra for lactose and lactose-ureide are shown in figures 2.4 and 2.5 respectively.
Common to both graphs is peak 1, which corresponds to the individual hydrogen atoms of lactose and lactose-ureide, and peak 2, which indicates the presence of hydroxyl groups. Peak 3 is unique to figure 2.5, and is taken to represent the NH_2 group on the urea moiety of lactose-ureide.

2.2.2.3 Low energy fast ion bombardment mass spectrometry (LFIB-MS)

Mass-spectrometry is a technique which allows the qualitative and quantitative identification of molecules based upon their mass. LFIB-MS was employed to analyse unlabelled lactose-ureide, lactose [^{13}C]ureide and lactose [$^{15}N^{15}N$]ureide. During LFIB-MS analysis, molecules are positively ionised with H^+ at a low energy level to prevent excessive fragmentation. A few mg of each sample were dissolved in glycerol and then introduced into the mass-spectrometer. A print-out of each analysis is shown in figures 2.6-2.8.
The peak at 385 MW in figure 2.6 corresponds to the molecular weight of the unlabelled lactose-ureide ion. Peaks at 223, 247, 283 and 345 MW are taken to indicate fragmentation products of lactose-ureide, of which the latter is probably lactose. The peaks at 386 and 387 MW in figures 2.7 and 2.8 correspond to the molecular weight of ionised lactose [^{13}C]ureide and lactose [$^{15}N^{15}N$]ureide respectively. Peaks at 277 and 369 MW in figures 2.7 and 2.8 are taken to represent three and four unit oligomers of the glycerol used in the analytical procedure, and the peak at 225 MW in both figures is likely to be a fragmentation product of lactose.

2.2.2.4 Electrospray mass spectrometry (ESMS)

ESMS was another mass spectrometry technique used to confirm the molecular weight of lactose [^{13}C]ureide and lactose [^{15}N^{15}N]ureide. ESMS is a relatively new and very sensitive technique which can measure the mass of a range of biomolecules, from amino acids to whole proteins. Mass can be determined very accurately (typically within 0.01%) and sample preparation is relatively straightforward (Mann and Wilm, 1995). The major advantage of this technique in the analysis of lactose-ureide is the lack of substrate fragmentation throughout the analytical process, resulting in a cleaner mass profile. This also allows an accurate measure of sample purity. A few milligrams of lactose [^{13}C]ureide and lactose [^{15}N^{15}N]ureide were dissolved separately in an acetonitrile/water solution (1:1, v/v) and then introduced into the spectrometer (VG Quattro II Electrospray Mass Spectrometer, Fison, UK). The two printouts obtained are shown in figures 2.9 and 2.10. Both figures show a large peak corresponding to the molecular weight of ionised lactose [^{13}C]ureide (386.1) and lactose [^{15}N^{15}N]ureide (387.1). Smaller peaks are apparent for ions with 1 or 2 extra mass units, and these are thought to represent naturally occurring isotopically labelled lactose-ureides species, probably containing ^{13}C atoms in the sugar moiety. The peaks at 408.0 and 409.0 in figures 2.9 and 2.10 respectively are taken to represent the sodium adduct of each lactose-ureide. Sodium ions are a common contaminant during laboratory procedures, and in this case, both lactose-ureides are thought to have been partially ionised with Na^+ rather than H^+

2.2.3 Analysis summary

Analysis of lactose and lactose-ureide with IR and NMR gives a clear indication of the chemical species which are common to both molecules (OH and H species), and those which are unique to lactose-ureide (C-N and C=O bonds within urea, and the peptide bond between urea and lactose). The mass-spectrometry analyses indicate dominant peaks for unlabelled lactose ureide, lactose [^{13}C]ureide, and lactose [^{15}N^{15}N]ureide at their corresponding molecular weights. Therefore, it is concluded that lactose-ureide, lactose [^{13}C]ureide, and

lactose [$^{15}N^{15}N$]ureide were all successfully synthesised through the method described in section 2.2.2.

2.2.4 Analysis of purity

In addition to successfully synthesising lactose-ureide, lactose [^{13}C]ureide, and lactose [$^{15}N^{15}N$]ureide, it was also necessary to determine the purity of each sample. A urea assay was used to detect any contamination of unreacted urea, while a further mass-spectrometry technique was employed to detect lactose and other contaminants. Lactose [$^{15}N^{15}N$]ureide was analysed for urea contamination, and unlabelled lactose-ureide for lactose contamination. The results obtained were taken to be representative of all three lactose-ureides.

2.2.4.1 Urea assay

The concentration of urea in samples of lactose [$^{15}N^{15}N$]ureide was measured by the Bertholot method (Kaplan, 1965) as described in section 2.4.3.2. A series of dilutions of lactose [$^{15}N^{15}N$]ureide in deionised water were analysed. Results are shown in table 2.1.

Table 2.1 Urea assay for five lactose [$^{15}N^{15}N$]ureide solutions of differing concentration

Sample dilution (g/l)	*Urea contamination (g/100g)*
1	- not detected -
2	not detected -
5	0.102
10	0.226
20	0.182

On average, the samples tested contained only 0.17 g (SD 0.06) urea/100 g sample.

2.2.4.2 Gas chromatography mass spectrometry (GCMS)

Samples of 1-2 mg lactose and unlabelled lactose-ureide were separately resolved using a gas-chromatography column (DB-1, J & W Scientific) before entering a mass spectrometer (VG TS-250, Fison, UK). Traces for lactose and unlabelled lactose-ureide are shown in figures 2.11 and 2.12 respectively. Peaks corresponding to different molecules are marked by their elution time. There are two major peaks in each figure (94 and 97 for lactose, 84 and 88 for lactose-ureide). The presence of two peaks for each sample most likely represents the two isomers of each molecule.

2.2.5 Analysis summary

The urea assay and GCMS analysis indicate only a low level of contamination by urea or lactose in each lactose-ureide sample tested. Additionally, the GCMS analysis suggests negligible contamination of substances other than lactose. This is reinforced by the ESMS analysis (section 2.2.2.4) which suggests no contamination of molecules under 250 MW in lactose [$^{15}N^{15}N$]ureide. In summary, it was concluded that the lactose-ureide samples tested were extremely pure under the conditions tested.

2.3 Clinical trial

A clinical trial was conducted on nine healthy adult volunteers (6 females and 3 males). The subjects were requested to consume the diet provided to them over five days. The diet was marginally adequate in protein, on average 36 (SD 2) g/d. The protein content of the diet was selected to enhance the rate at which urea-N would be hydrolysed by the colonic microflora, whilst being sufficient to maintain nitrogen homeostasis in normal man (Langran *et al*, 1992). Energy content of the diet was estimated to satisfy the requirement for each individual, about 1.5 x basal metabolic rate (BMR), and was in the region of 40 kcal/kg/d, similar to that employed by Giordano *et al* (1968). Subject characteristics and details of the diet are detailed in this section. All food was purchased at Sainsbury's

Supermarket, and the protein and energy content were calculated from the information provided on the packaging.

2.3.1 Subjects

Nine adult subjects were recruited from the Department of Human Nutrition, Southampton. They agreed to participate and signed a consent form after the nature of the study had been explained. All subjects were highly motivated individuals and in good health at the time of the study. A letter describing the study was also sent to each subject's General Practitioner. The protocol had the approval of the Southampton Hospital and South West Hampshire Health Authority Ethical Sub-committee. Sex, age and the body-mass index (BMI) for each subject is shown in table 2.2. Metabolic studies were conducted at the Clinical Nutrition and Metabolism Unit, Southampton General Hospital.

Table 2.2 Sex, age and BMI for nine healthy adult subjects consuming a diet marginally adequate in protein.

Subject	*Sex*	*Age*	*BMI*
1	M	23	20.5
2	F	23	22.4
3	F	23	18.9
4	F	19	20.6
5	F	23	23.0
6	F	25	22.1
7	M	26	25.0
8	F	26	20.8
9	M	23	22.1

2.3.2 Diet

Food was prepared and provided to each subject daily for the duration of the study. In this way, complete control over the dietary intake was assured. The duration of feeding was deemed to be sufficient to allow subjects to

accommodate the lowered protein diet and attain nitrogen equilibrium (Langran *et al*, 1992). The diet plan was structured around three meals each day plus snacks (Table 2.3), except during the metabolic investigations on day four, when six meals of roughly equal nitrogen and energy content were given three-hourly.

Table 2.3 Basic diet plan for nine healthy adult subjects consuming a diet marginally adequate in protein

Breakfast	Frosted rice cereal; Full-fat milk; Fruit Juice
Lunch	Salad sandwich with full-fat mayonnaise dressing; Potato crisps; Carbonated beverage; Fruit
Dinner	Pre-prepared meal e.g. Vegetable lasagne; Mixed salad with full-fat mayonnaise dressing; Tinned fruit; Double cream
Snacks	Sponge biscuits; Fruit sweets

The energy content of the diet was adjusted to meet individual requirements with the addition of appropriate amounts of carbohydrate (carbonated beverages, fruit sweets) and fat (mayonnaise, cream). Subjects were allowed to satisfy their appetite by consuming additional energy as needed, mainly as refined carbohydrate. The contribution of these foodstuffs was calculated using Food Composition Tables (McCance & Widdowson, 5th Edition). Subjects were asked to recorded any foods not consumed. Foodstuffs which are known to contain high natural enrichment of ^{13}C such as cane sugar, corn products and tropical fruit were avoided as much as possible so as to reduce to a minimum variation in the background enrichment of ^{13}C. A complete five-day diet plan for subject 1 is detailed in appendix 2B. Overall compliance to the diet was excellent. Only one subject failed to consume all the food provided on day 1.

2.4 Biochemical analyses

This section describes the biochemical techniques that were used during the experimental work of the thesis. All methods had been previously developed and used by other workers at the Institute of Human Nutrition, Southampton, UK, and the Children's Nutrition Research Centre, Houston, TX, USA.
Details of all reagents are given in appendix 2C.

2.4.1 Breath CO_2 excretion rate

The rate of excretion of CO_2 on the breath was measured with an indirect calorimeter using a ventilated hood system (Deltatrac, Datex Instrumentarium Corporation, Helsinki, Finland). The duration of each measurement was ten minutes, and this was taken to be representative of total CO_2 excretion over the hour. Measurements were made while the subjects were lying relaxed on a bed. Subjects were asked to restrict movement as much as possible over the period of study so as not to increase CO_2 excretion rate through unnecessary exercise.

2.4.2 Isotopic enrichment of breath CO_2

Subjects exhaled mid- to late-expired air into a bag (Quintron, Milwaukee, USA) and 10 ml aliquots of each breath sample were transferred in triplicate to evacuated glass gas tubes (Europa Scientific, Crewe, UK) to await analysis. Isotopic enrichment of breath CO_2 was determined by isotope-ratio mass-spectrometry (ABCA system, Europa Scientific Ltd, Crewe, England).

2.4.3 Ammonia and urea concentration in urine

The ammonia and urea concentrations of urine samples were determined according to the Bertholot method (Kaplan, 1965) utilising a newly developed small-scale analysis. This analysis has been recently validated (T. Meakins, PhD Thesis). Reagents are detailed in appendix 2C.

2.4.3.1 Ammonia assay

100 µl aliquots of urine and standards were added to 900 µl deionised water in separate glass test tubes. 10 µl of each solution were pipetted in triplicate to separate microtitre plate wells. 100 µl of working PNP (phenol nitroprusside) solution were subsequently added to each well, followed by 100 µl of working alkaline hypochlorite solution. The plate was incubated in a water bath at 37°C for 20 min and then the absorption of samples at 550 nm was determined (Biotek EL 340 microplate biokinetics reader).

2.4.3.2 Urea assay

100 µl aliquots of urine were added to 1.9 ml of deionised water in separate glass test tubes. 25 µl aliquots of these diluted samples plus standards were added to labelled glass test tubes in duplicate. 1 ml working urease solution was added to each tube, and these were then incubated in a water bath at 37°C for 20 min. 20 µl of each sample and standard were pipetted in triplicate into separate wells of a microtitre plate. 100 µl working PNP solution were added to each well followed by 100 µl alkaline hypobromite solution. The microtitre plate was incubated for 20 min at 37°C in a waterbath and absorption at 550 nm was determined.

2.4.4 Isotopic enrichment of urinary urea

Urinary urea was isolated by short column ion-exchange chromatography (Jackson *et al*, 1980). The volume of a urine sample containing about 2 mg urea nitrogen was calculated from data obtained from an ammonia and urea assay, section 2.4.3. Urine samples were adjusted to pH 2 in separate glass test tubes with concentrated HCl solution, using cresol red as an indicator. Samples were loaded onto glass columns, containing a washed resin up to a height of about one inch (Dowex 50-WX8 - 200 Mesh, H^+ form), and allowed to elute. Each column was washed with 2 ml deionised water. 10 ml citrate buffer were then added to each column and allowed elute. A further 15 ml citrate buffer were added, and allowed to elute into separate 50 ml glass conical flasks.

Samples were adjusted to pH 12 using 40g/100g sodium hydroxide solution. A few anti-bumping granules were added to each flask, and the sample volumes were reduced to approximately 1 ml using a hot plate. Concentrated samples were transferred to plastic tubes, sealed with a plastic bung, and stored at -70°C until further analysis. Nitrogen gas was liberated from the samples from the addition of about 1 ml lithium hypobromite solution to the samples in clean glass tubes. The liberated nitrogen gas was collected into evacuated glass tubes. The isotopic ratio of [$^{15}N^{15}N$]- and [$^{14}N^{15}N$]urea to unlabelled urea was determined by isotope-ratio mass-spectrometry (VG Isogas, Middlewich, Cheshire, England)

2.4.5 Blood plasma preparation

Whole blood was collected into universal tubes containing 1 ml isomolar NaCl / EDTA solution and immediately spun in a centrifuge for 10 to 12 min at 3000 rpm, 4°C (Sorvall RT6000 Refrigerated Centrifuge). The resultant plasma was transferred to universal tubes containing 50 µl of enzyme inhibitor and stored at -70°C until further analysis.

2.4.6 Plasma VLDL apoB-100 isolation

10 ml samples of a saline EDTA solution were pipetted into polycarbonate centrifuge bottles (Beckman Instruments Inc., Palo Alto, CA, USA) and carefully underlayed with a 10 ml plasma sample. Samples were spun in a centrifuge (Beckmann Ultracentrifuge using a Ti70.1 TY rotor) for 16 h at 40,000 rpm, 15°C (Egusa *et al*, 1983). The tubes were held up against a black background and the floating cloudy layer of VLDL was removed into pre-weighed 12 ml tapered plastic centrifuge tubes. An equal amount (g) of isopropanol was added to each sample, and the tubes were vortexed for 5 min and left covered overnight at room temperature to allow precipitation of apoB-100. Samples were spun down in a centrifuge for 30 min at 6,000 rpm, and the supernatant was discarded. The apoB-100 precipitates were transferred to FBG Trident glass vials with a teflon lined screw cap (Anachem, Bedford, UK) and stored at -20°C before acid hydrolysis.

2.4.7 Plasma fibrinogen isolation

100 ul aliquots of a 0.025M $CaCl_2$ solution were added to separate 250 µl plasma aliquots in eppendorf tubes, and mixed by vortex. 100 µl thrombin solution (100 units/ml) were added, and each sample was gently stirred with a wooden applicator stick. After 2 to 3 hr at 4°C, a clot has formed and adhered to each applicator stick, and this was removed to a clean eppendorf tube. To wash the clot, 0.5 ml 1.14 M NaCl solution was added to each tube, and mixed by vortex. Samples were centrifuged (Eppendorf S414 centrifuge) for 5 min, and the supernatant discarded. Each clot was washed twice more in this way. Samples were then transferred to FBG Trident glass vials and stored at -20°C before acid hydrolysis (Section 2.4.9).

2.4.8 Plasma albumin isolation

100 µl of a 10 g/100g TCA solution were added to 100 µl of each plasma sample in separate eppendorf tubes which were kept on ice. Tubes were mixed by vortex and centrifuged for 10 min. The supernatant was removed and the precipitate washed three times with 250 µl of the TCA solution. One drop of the TCA solution was then added to each sample, followed by 1 ml absolute ethanol. The tubes were mixed by vortex to dissolve the precipitate, and then centrifuged for 10 min. The supernatant was transferred to FBG Trident glass vials and dried under vacuum. Samples were stored at -20°C before acid hydrolysis (Section 2.4.9).

2.4.9 Acid hydrolysis

1 ml 6M hydrochloric acid solution (ARISTAR grade, BDH-Merck) containing 1 ml/l phenol and degassed by bubbling N_2 through the solution was added to each sample tube. Tubes were capped tightly, sealed with PTFE tape, and placed in an oven at 110°C for 24 hr. Samples were dried under vacuum and stored at -20°C until further analysis.

2.5 Theoretical considerations

2.5.1 *Theoretical model for lactose [^{13}C]ureide metabolism*

Lactose [^{13}C]ureide was given to nine healthy adults consuming a diet marginally adequate in protein. The aim of this part of the clinical trial was to determine the extent to which lactose-ureide is fermented and hydrolysed in the colon. The theoretical model of lactose [^{13}C]ureide metabolism is shown in figure 2.13. Lactose [^{13}C]ureide given orally in presumed to pass through the upper GI tract intact. When it enters the colon, it is fermented by the bacteria to form lactose and [^{13}C]urea. The [^{13}C]urea part is then available for further hydrolysis, forming NH_3 ions and labelled $^{13}CO_2$. Label may exit the colon through three routes.

2.5.1.1 Retention of $^{13}CO_2$ in the body

$^{13}CO_2$ may be absorbed from the colon and retained in a compartment of the body's bicarbonate pool, or moved to other metabolic pathways. It has been postulated that the body can be viewed as containing three bicarbonate pools which CO_2 may enter: a rapidly turning over central vascular pool which includes circulating CO_2; a rapidly turning over peripheral pool, including organs such as the heart, brain and liver ;and a more slowly turning over peripheral pool, including muscle, adipose tissue, and bone (Irving *et al*, 1983). Therefore, isotopically labelled CO_2 entering a compartment of the body bicarbonate pool with a slow turnover rate may not be excreted on the breath over a relatively short period of measurement.

$^{13}CO_2$ absorbed from the colon may also be incorporated into a number of metabolic pathways. Carbomyl phosphate, a precursor in urea synthesis, is formed in the liver from CO_2 and an ammonium ion through carbomyl phosphate synthetase. During an IV infusion of radioactively labelled $NaH^{14}CO_3$, Elia and colleagues (1992) reported that 84% of urinary label was in the form of urea, suggesting synthesis of carbomyl phosphate from $^{14}CO_2$. Acetyl CoA is combined with CO_2 to form malonyl CoA, which may then be used in the

de novo synthesis of fatty acids, or to elongate fatty acid chains. In gluconeogenesis, pyruvate combines with CO_2 through pyruvate carboxylase to form oxaloacetate, and CO_2 also appears to be consumed during the synthesis of Vitamin K.

2.5.1.2 Excretion of ^{13}C in stool

A further route through which ^{13}C may be lost from the body is through the stool. The form in which label may be lost is not fully known, and it may include intact lactose [^{13}C]ureide and [^{13}C]urea, $^{13}CO_2$, and products of bacterial metabolism. A previous study has shown that detectable levels of urea were never found in the faecal dialysate from normal subjects, suggesting that any urea in the colon would normally be hydrolysed by the bacteria (Wrong *et al*, 1970).

2.5.1.3 Excretion of $^{13}CO_2$ on breath

$^{13}CO_2$ which enters the body and is not incorporated into other metabolic pathways is excreted on the breath. The extent of this excretion has been extensively studied in different metabolic states, using labels such as $NaH^{13}CO_3$. In different studies, it has been observed that between 51% and 95% of administered ^{13}C label is excreted on the breath as $^{13}CO_2$ under various methodological conditions (for a review, see El-Khoury *et al*, 1994*b*).

For the present purpose, it is assumed in the model that the amount of ^{13}C label retained in the body as $^{13}CO_2$, or excreted in the stool in some form, is negligible. Therefore, by measuring the amount of $^{13}CO_2$ excreted on the breath, we can estimate the extent to which lactose [^{13}C]ureide is fermented and hydrolysed in the colon.

2.5.2 Dose of lactose [^{13}C]ureide

A single oral dose of 500 mg of lactose [^{13}C]ureide was administered to subjects. The total amount of label excreted on the breath over 48 h was measured in order to determine the extent to which lactose-ureide is fermented and hydrolysed in the gut, and to obtain a profile of $^{13}CO_2$ excretion. It was considered that the amount of label given would be sufficient to enrich breath CO_2 to a level which could be measured with reliability by isotope-ratio mass-spectrometry, as calculated from a previous study (Murphy *et al*, 1995), taking into account variations in background $^{13}CO_2$ enrichment.

2.5.3 Calculating the amount of breath $^{13}CO_2$ excretion

The amount of $^{13}CO_2$ excreted on the breath as a percentage of the dose given was calculated for each sample as follows, using information on the rate of CO_2 production (section 2.4.1) and the level of breath $^{13}CO_2$ enrichment (section 2.4.2)

$$\% \text{ administered dose excreted per h} = \left(\frac{\text{mmol excess } ^{13}CO_2 \text{ per mmol } CO_2}{\text{mmol } ^{13}C \text{ administered}}\right) \times \text{mmol } CO_2 \text{ excreted per h} \times 100$$

$$\text{mmol excess } ^{13}CO_2 \text{ per mole } CO_2 = \frac{\left(^{13}C_t - ^{13}C_{t0}\right) \times Ref_{PDB}}{1000}$$

where

$^{13}C_t$	is the sample ^{13}C enrichment at time t
$^{13}C_{t0}$	is the sample ^{13}C enrichment at time t0 (baseline)
Ref_{PBD}	is the ^{13}C isotopic abundance of a reference material, Pee Dee Belemnite (0.0112372 APE)

When values for *mmol CO_2 excreted per h* were not measured directly, a value which was taken at a similar time point during the period of measurement was used in the calculation as a best estimate.

Total excretion of $^{13}CO_2$ on breath over 48 h as a percentage of the dose given was calculated from the area under the curve according to Simpsons Rule. This is calculated as follows:

$$\text{Area} = \frac{\text{interval width}}{3}\left[(\text{first} + \text{last ordinate}) + 4\left(\sum \text{evens}\right) + 2\left(\sum \text{odds}\right)\right]$$

2.5.4 Theoretical model for lactose [$^{15}N^{15}N$]ureide metabolism

Lactose [$^{15}N^{15}N$]ureide was given to nine healthy adults consuming a diet marginally adequate in protein in order to determine the fate of ^{15}N and quantify the retention of urea nitrogen salvaged in the colon. The theoretical model of lactose [$^{15}N^{15}N$]ureide metabolism is shown in figure 2.14. Lactose [$^{15}N^{15}N$]ureide given orally is presumed to pass through the upper GI tract intact. When it enters the colon, it is fermented to release lactose and [$^{15}N^{15}N$]urea. The urea part is then available for hydrolysis, forming CO_2 and labelled $^{15}NH_3$ ions. Label may exit the colon through three routes.

2.5.4.1 Excretion of ^{15}N in the stool

Label may exit the colon through the stool. The form in which label occurs in the stool is not known, but is expected to be similar for lactose [$^{15}N^{15}N$]ureide as for lactose [^{13}C]ureide i.e. as intact lactose-ureide, urea, and products of bacterial metabolism.

2.5.4.2 Excretion of ^{15}N in the urine

[$^{15}N^{15}N$]urea released from the hydrolysis of lactose [$^{15}N^{15}N$]ureide may be absorbed directly from the colon, and excreted as urinary [$^{15}N^{15}N$]urea.

Additionally, $^{15}NH_3$ ions released from urea hydrolysis may be absorbed from the colon and used to reform urea. This will be excreted as [$^{14}N^{15}N$]urea

2.5.4.3 Retention of ^{15}N in the body

Label which is not recovered in the stool or as urinary urea is presumed to have been retained within the body's metabolic nitrogen pool in some form. Potentially, this is considered to include essential and non-essential amino acids synthesised *de novo* by the colonic bacteria.

By measuring the excretion of ^{15}N into the stool and urinary urea, it should be possible to calculate the proportion of label retained by the body.

2.5.5 Dose of lactose [$^{15}N^{15}N$]ureide

Lactose [$^{15}N^{15}N$]ureide was given orally as a *prime and intermittent* doses during the measurement period. The aim of this dose regimen was to achieve a state of isotopic equilibrium over the study period, and so be able to calculate the rate of excretion of label into the urine as a proportion of the rate of administration, and hence quantify the exit of ^{15}N label from the colon. A prime dose was given to reduce the time necessary to achieve a steady isotopic state. The prime dose was 3.21 mg/kg, with 3 hourly intermittent doses of 0.64 mg/kg for 15 h. The doses were calculated from a preliminary study detailed below.

2.5.6 Preliminary study

One of the important applications of developing a new non-invasive method to quantify the colonic salvage of urea nitrogen will be to assess the utilisation of *de novo* synthesised lysine by the body. With this in mind, the preliminary study had two aims. The first was to identify a plasma protein which would reach a steady isotopic state within a time period compatible with the clinical trial. The second was to calculate the dose of lactose [$^{15}N^{15}N$]ureide needed to cause measurable changes in ^{15}N enrichment in the lysine fraction of that protein. After calculating a suitable dose of label, samples of blood could then be taken from

subjects during a clinical trial, to be later analysed for [^{15}N]lysine enrichment. The protocol was as follows.

The study was carried out in one healthy male adult (age 22 years) at the Children's Nutrition Research Centre in Houston, Texas, USA. 6 µmol [^{15}N]lysine/kg/hr (99 % APE, Cambridge Isotope Laboratories, USA) were administered to the subject for 12 h in one hourly oral doses. Hourly isonitrogenous and isoenergetic meals consisting of Ensure Plus (Ross Laboratories, Cleveland, OH) and Fortical (Cow & Gate, Trowbridge, UK) were given, supplying 0.8 g protein/kg/d and 125 kJ/kg/d. A 10 ml blood sample was taken at time zero, and thereafter every two hours for 12 hours. ApoB-100, fibrinogen, and albumin were isolated from the plasma according to the procedures described in sections 2.4.6 to 2.4.8.

ApoB-100 samples were hydrolysed with 1 ml 6M HCl and then purified using a disposable two-piece column (Fisher Scientific) containing resin (AG-50W-X8, 100-200 mesh, H+ form). 0.5 ml esterification reagent (5 ml propanol : 1 ml acetyl chloride) was added to purified hydrolysates and mixed by vortex. Samples were dried with heat under nitrogen. 100 µl derivatisation reagent (heptafluorobutyricanhydride) were added to each sample and mixed by vortex. Samples were heated at 60°C for 20 min, and then cooled and dried under nitrogen. 400 µl ethyl acetate were added to each sample and mixed. Samples were transferred to crimp autosampler vials which were then capped before analysis. The ^{15}N isotopic enrichment of the lysine fraction was determined by gas-chromatography mass-spectrometry (Hewlett-Packard 5988A GCMS system using a DB-5 column).

Fibrinogen and Albumin samples were directly analysed for isotopic enrichment using combustion isotope-ratio mass-spectrometry (ANCA system, Europa Scientific, Crewe, UK). This method of analysis was chosen because the anticipated change in total ^{15}N enrichment was not great enough to be measured accurately using gas-chromatography mass-spectrometry. Changes in [^{15}N]lysine enrichment were calculated by multiplying the values for total ^{15}N

enrichment by 20, which assumes an equal distribution of amino acids within each protein.

Results for apoB-100, fibrinogen, and albumin are shown in figure 2.15 (Appendix 2A). Isotopic equilibrium in the lysine fraction of plasma VLDL apoB-100 was achieved within 6 hr, and the plateau enrichment corresponded to approximately 5 atoms percent excess (APE). Lysine from both fibrinogen or albumin achieved a low level of isotopic enrichment during the study period, but neither appeared to achieve isotopic plateau. Therefore, apoB-100 appeared to be a suitable protein for quantifying the flow of salvaged urea nitrogen into lysine.

Using gas-chromatography mass-spectrometry (GCMS), the minimum change in ^{15}N enrichment that can be measured with accuracy is about 0.5 APE. This would mean giving dose of 0.3 μmol lactose [$^{15}N^{15}N$]ureide/kg/hr to achieve a similar enrichment in the lysine fraction of apoB-100, assuming that all ^{15}N administered is incorporated into lysine through the colonic microflora, and then absorbed by the body. A dose of 6 μmol lactose [$^{15}N^{15}N$]ureide/kg/hr allows for an equal distribution of label over 20 amino acids. Other considerations which need to be taken into account are: hydrolysed urea nitrogen which is absorbed as $^{15}NH_3$ ions, and either reformed to urea or fixed into amino acids, and label which may be lost through the stool. Therefore, it was concluded that the dose of lactose [$^{15}N^{15}N$]ureide needed to detect changes in ^{15}N enrichment in the lysine fraction of apoB-100 using GCMS was too large to be methodologically or economically practical. Combustion isotope-ratio mass-spectrometry (c-IRMS) can detect changes in ^{15}N isotopic enrichment as low as 0.0002 APE, although a larger sample size is required (Preston and Slater, 1994). By employing c-IRMS as the method of determining changes in ^{15}N enrichment in the lysine fraction of apoB-100, the dose of lactose [$^{15}N^{15}N$]ureide needed was considerably less than if GCMS was the method of analysis of isotopic enrichment. By considering all the factors discussed in this section, values for the prime and intermittent doses of lactose [$^{15}N^{15}N$]ureide were derived.

2.5.7 Calculations for ^{15}N excretion and retention

2.5.7.1 Urinary [$^{15}N^{15}N$]urea excretion

$$\text{Proportion of dose excreted} = \frac{\text{Rate of excretion of label}\left(\text{mg h}^{-1}\right)}{\text{Rate of administration of label}\left(\text{mg h}^{-1}\right)} \times 100$$

$$\text{Rate of excretion of label}\left(\text{mg h}^{-1}\right) = \frac{[^{15}\text{N}^{15}\text{N}]\text{urea}}{[^{14}\text{N}^{14}\text{N}]\text{urea}} \times \text{urea -N}\left(\text{mg h}^{-1}\right)$$

where

[$^{15}N^{15}N$]urea / [$^{14}N^{14}N$]urea	is the ratio of [$^{15}N^{15}N$]urea to unlabelled urea in urine at isotopic plateau
urea-N (mg h^{-1})	is the rate of excretion of urea nitrogen in the urine per hour, calculated from the total urea nitrogen excreted over 18 hours in mg
Rate of administration of label (mg h^{-1})	is the rate of administration of ^{15}N as lactose [$^{15}N^{15}N$]ureide per hour, derived from the intermittent dose divided by three

2.5.7.2 Urinary [$^{14}N^{15}N$]urea excretion

The calculation for the proportion of dose excreted as urinary [$^{14}N^{15}N$]urea was assumed to be similar as that for [$^{15}N^{15}N$]urea. Where isotopic plateau was not achieved, the highest value for the ratio of [$^{14}N^{15}N$]urea to unlabelled urea in urine was used to calculate the minimum proportion of dose excreted.

2.5.7.3 Stool ^{15}N excretion

Due to the long transit time of substance from mouth to anus, it was not appropriate within the study protocol to try to achieve ^{15}N isotopic equilibrium in the stool compartment. Hence, the proportion of ^{15}N dose excreted in the stool was calculated assuming a *single dose* of lactose [$^{15}N^{15}N$]ureide, being the sum of the prime and intermittent doses.

$$\%\text{ dose excreted in stool} = \sum\left(\frac{^{15}\text{N APE} \times \text{total stool N}}{\text{Total } ^{15}\text{N administered}}\right) \times 100$$

where

^{15}N APE x total stool N	is the isotopic enrichment of ^{15}N in each stool sample over baseline, multiplied by the total nitrogen content of that stool sample
$\sum(^{15}N$ APE x total stool N)	is the sum of all stools collected for that subject
total ^{15}N administered	is the total amount of lactose [$^{15}N^{15}N$]ureide administered, being the sum total of prime and intermittent doses (mg)

Appendix 2A Figures and Diagrams

Figure 2.1 Molecular structure of lactose-ureide showing proposed enzymatic cleavage sites

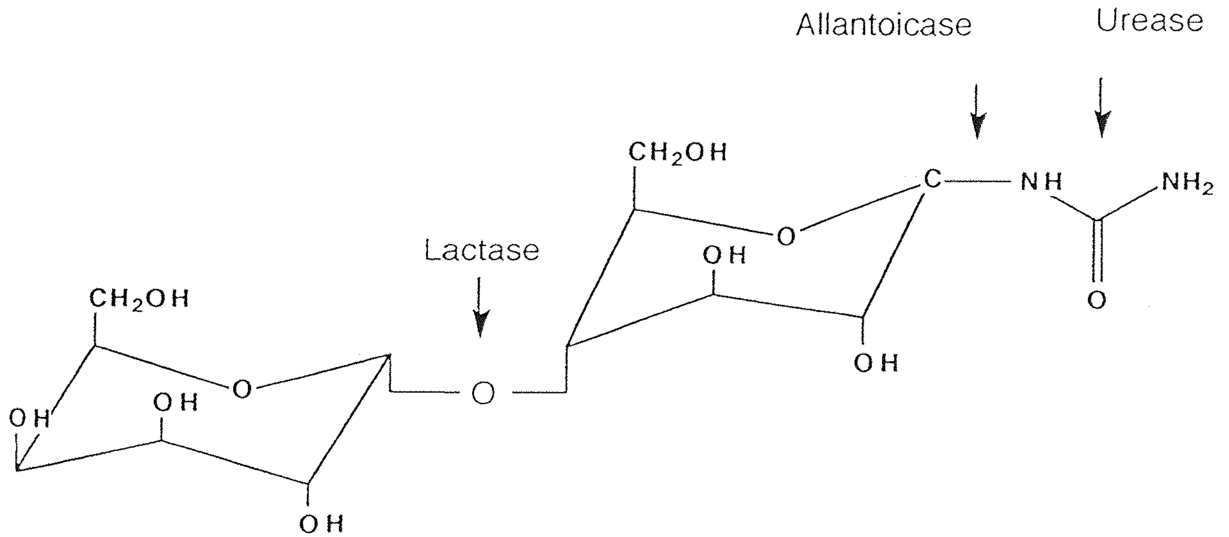


Figure 2.2 Infra-red (IR) spectrograph for lactose

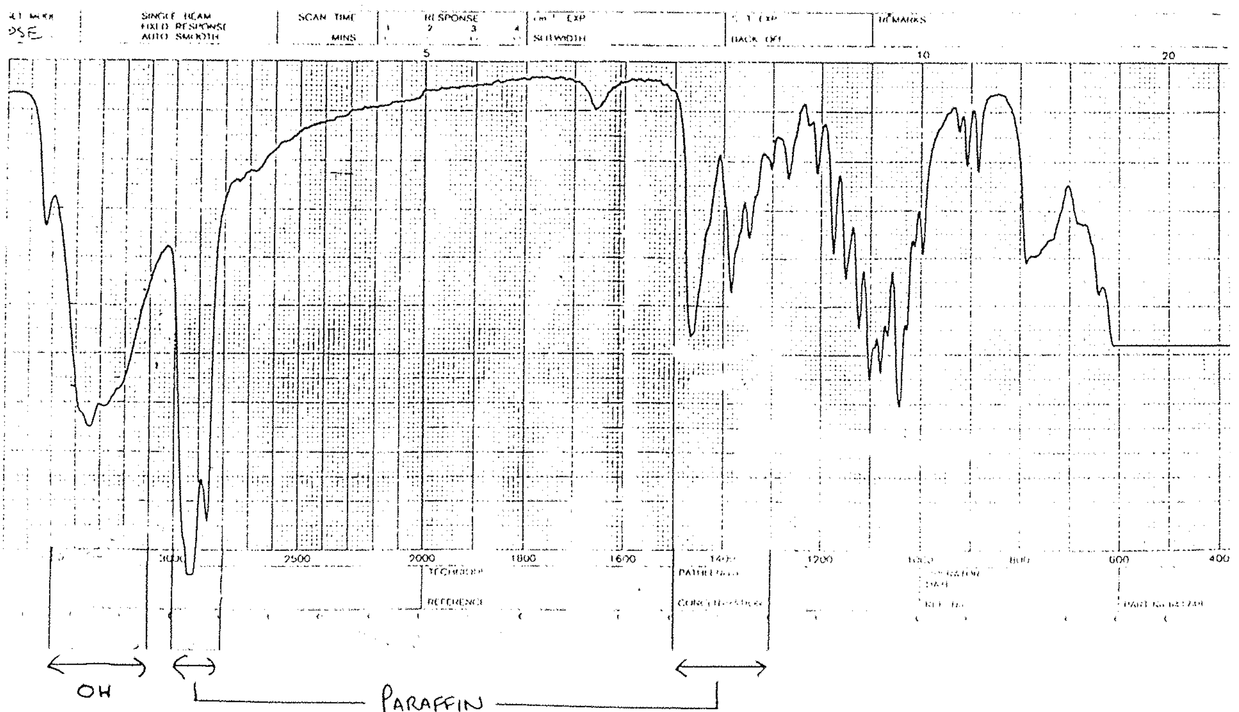


Figure 2.3 Infra-red (IR) spectrograph for lactose-ureide

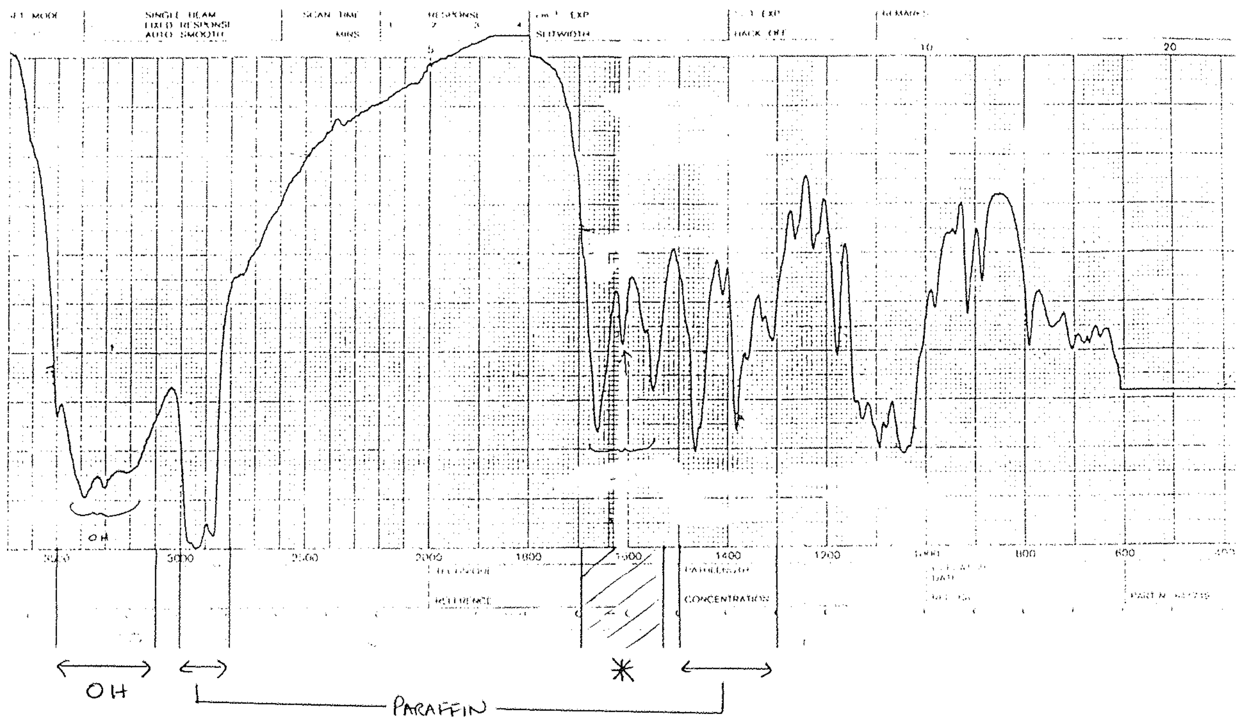


Figure 2.4 Nuclear magnetic resonance (NMR) spectrograph for lactose

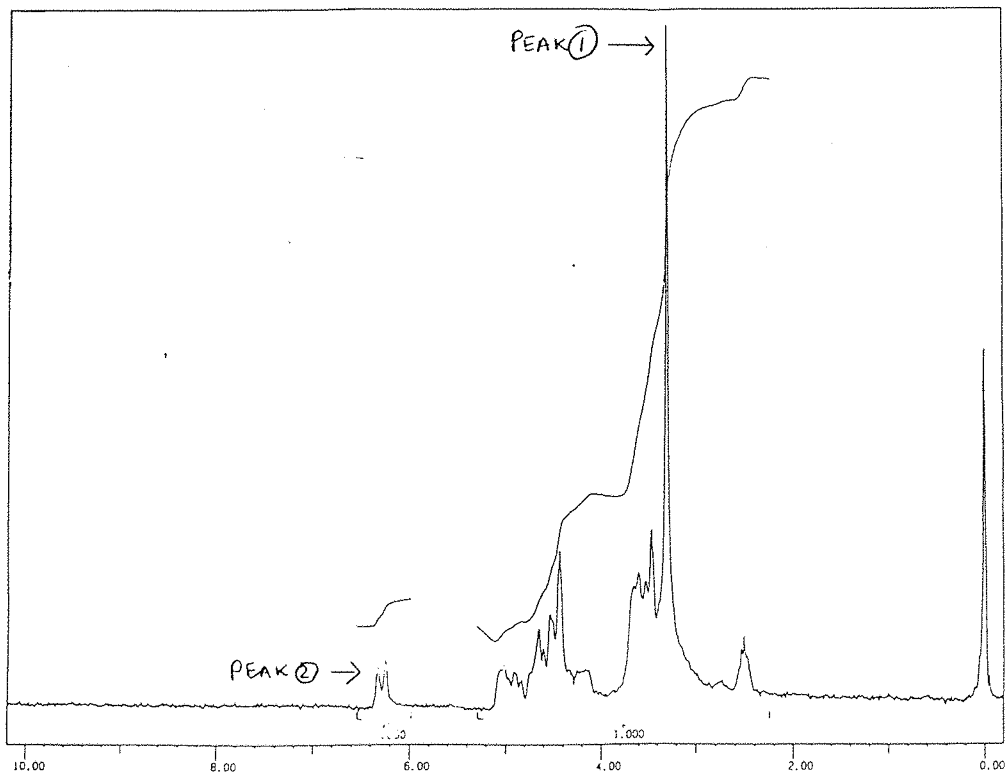


Figure 2.5 Nuclear magnetic resonance (NMR) spectrograph for lactose-ureide

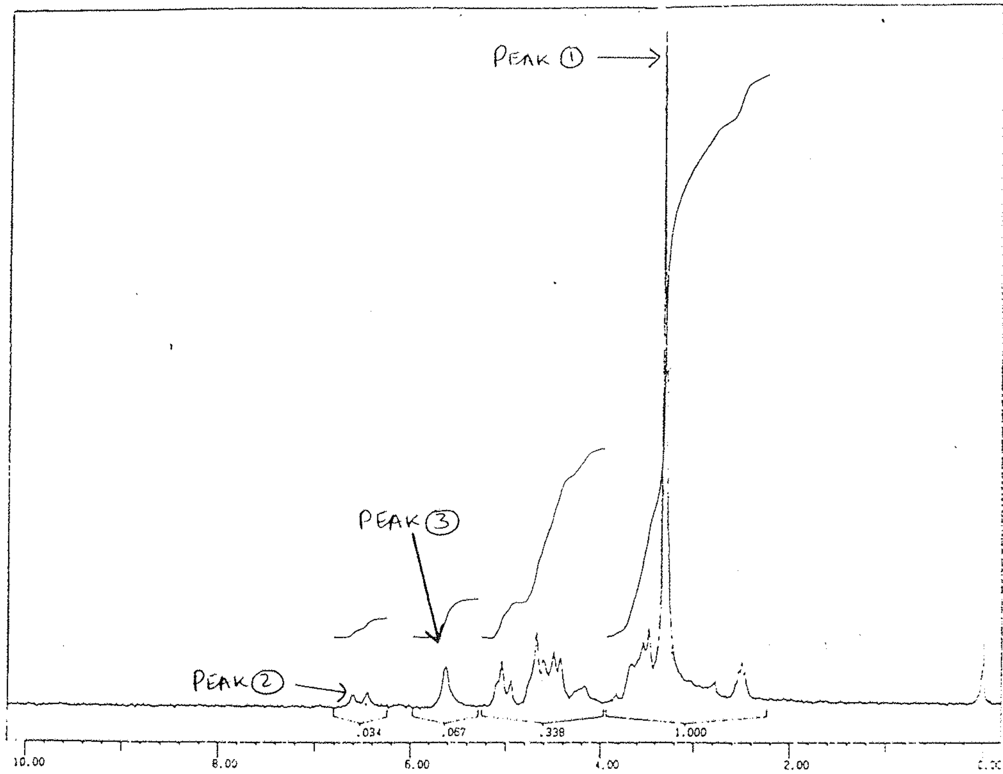


Figure 2.6 Low energy fast ion bombardment mass spectrograph (LFIB-MS) for lactose-ureide

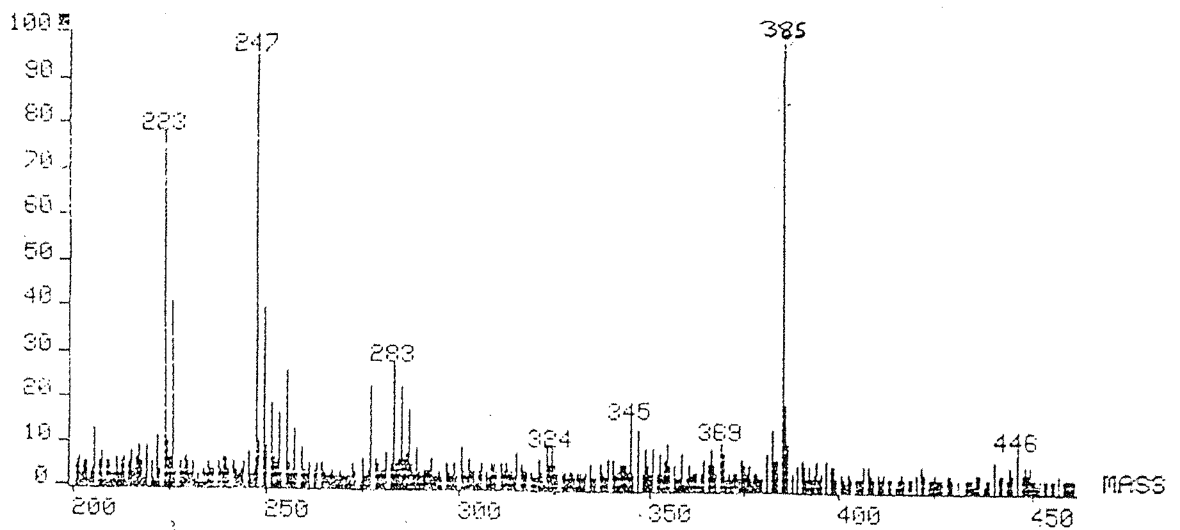


Figure 2.7 Low energy fast ion bombardment mass spectrograph (LFIB-MS) for lactose [^{13}C]ureide

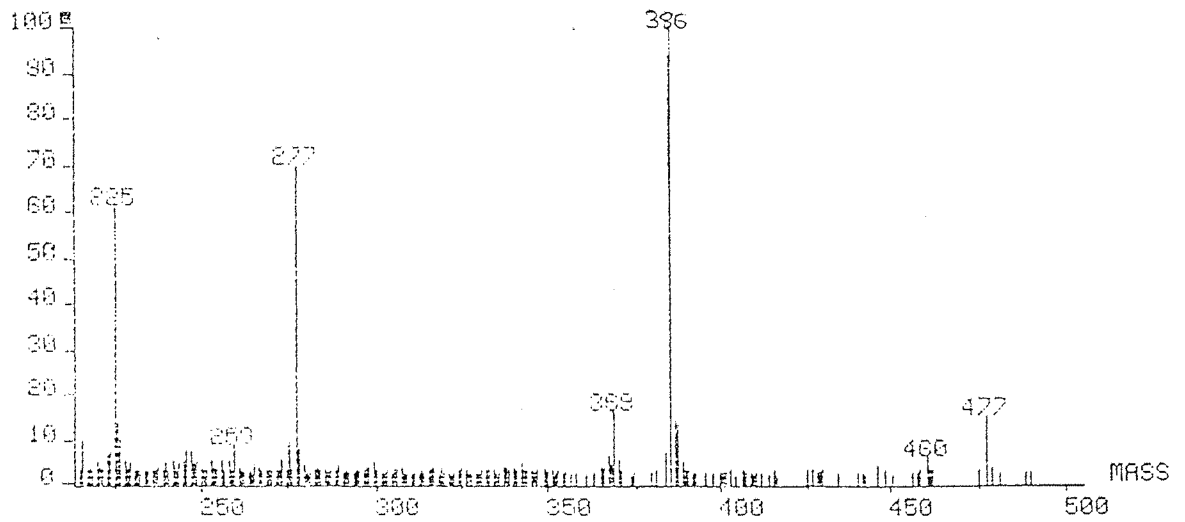


Figure 2.8 Low energy fast ion bombardment mass spectrograph (LFIB-MS) for lactose [^{15}N^{15}N]ureide

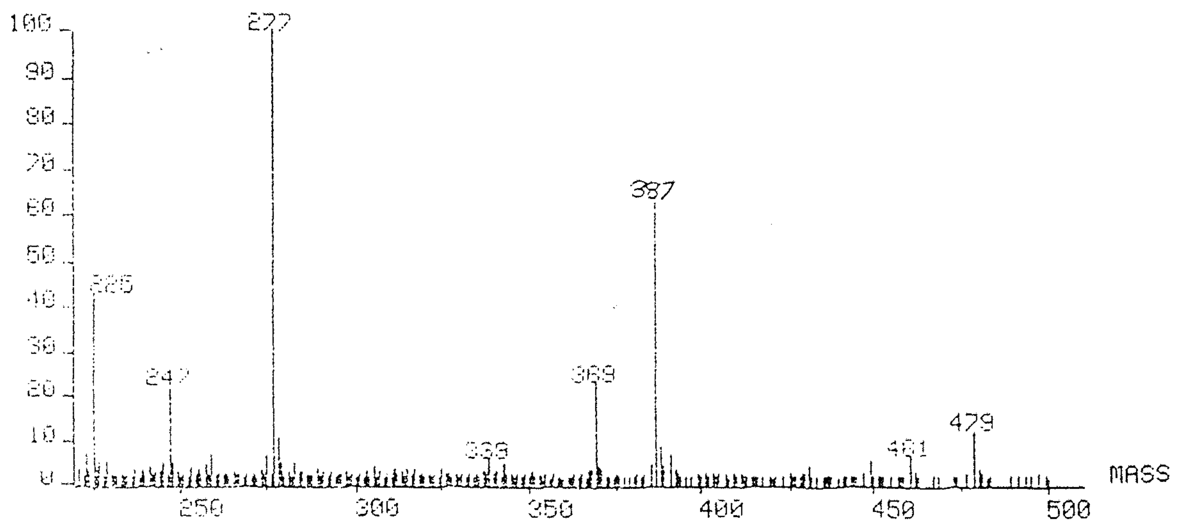


Figure 2.9 Electrospray mass spectrograph (ESMS) for lactose [^{13}C]ureide

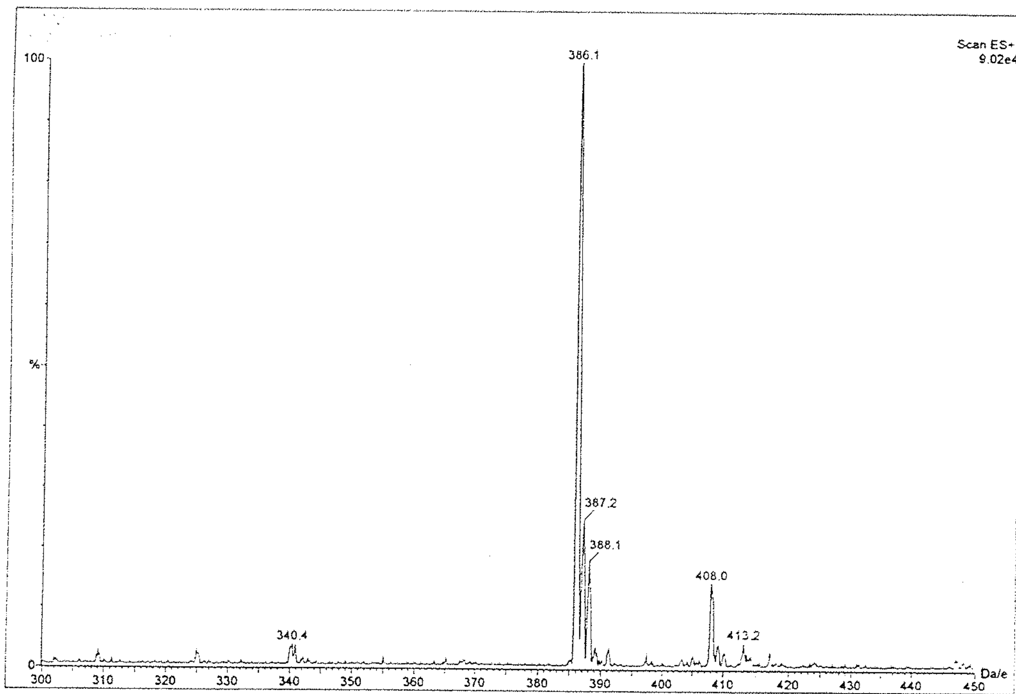


Figure 2.10 Electrospray mass spectrograph (ESMS) for lactose [^{15}N^{15}N]ureide

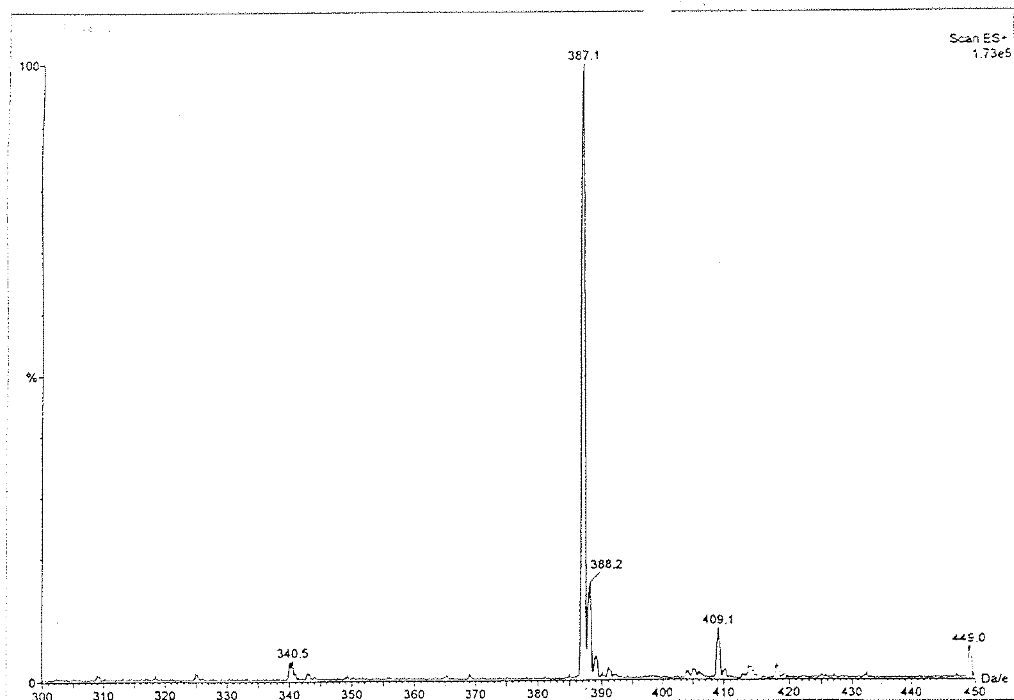


Figure 2.11 Gas chromatography mass spectrograph (GCMS) for lactose

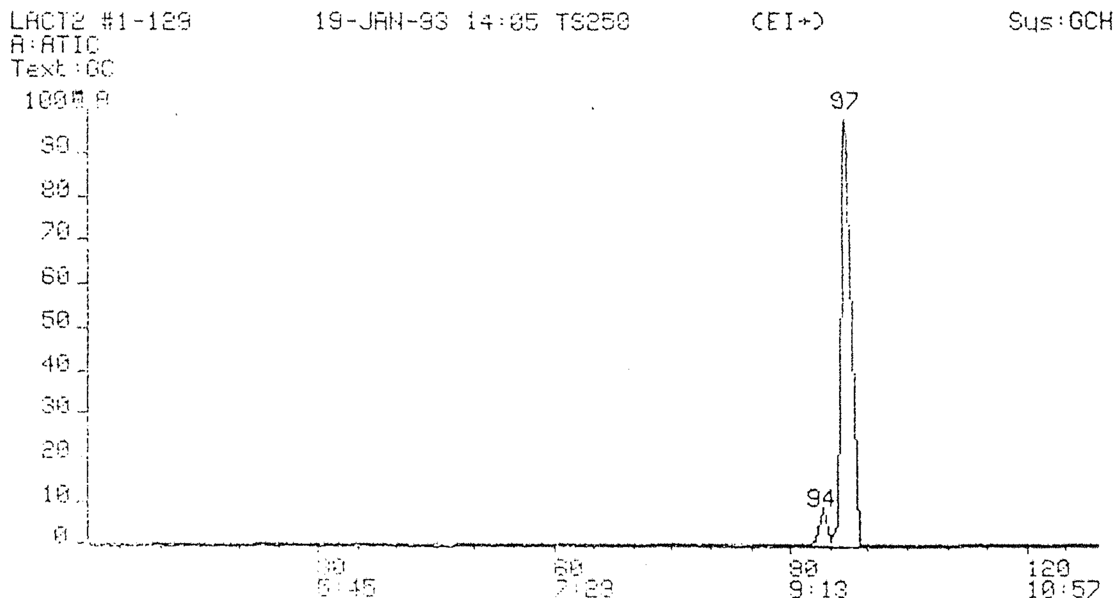


Figure 2.12 Gas chromatography mass spectrograph (GCMS) for lactose-ureide

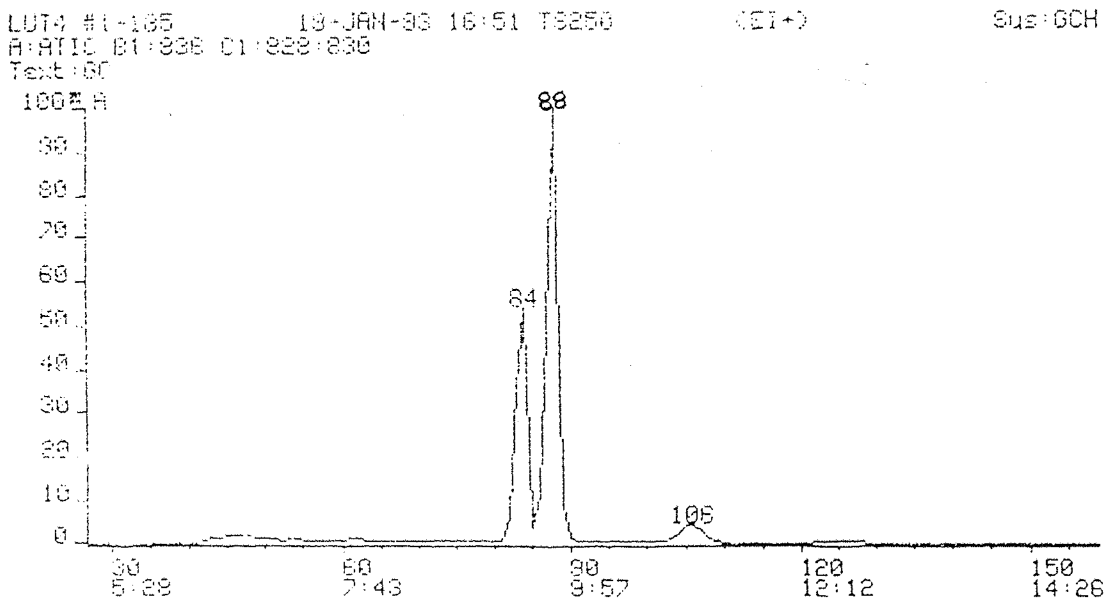


Figure 2.13 Proposed model of lactose [^{13}C]ureide metabolism

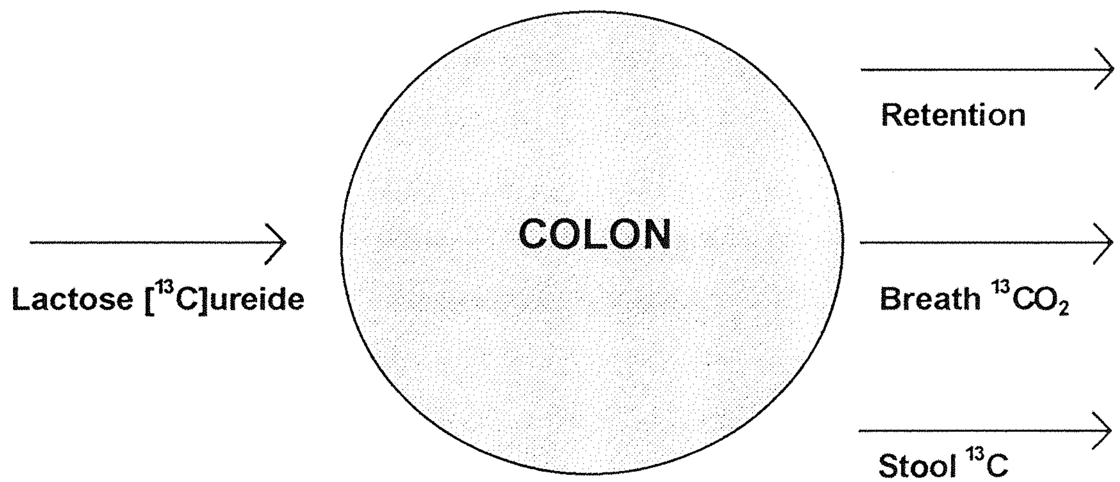


Figure 2.14 Proposed model of lactose [^{15}N^{15}N]ureide metabolism

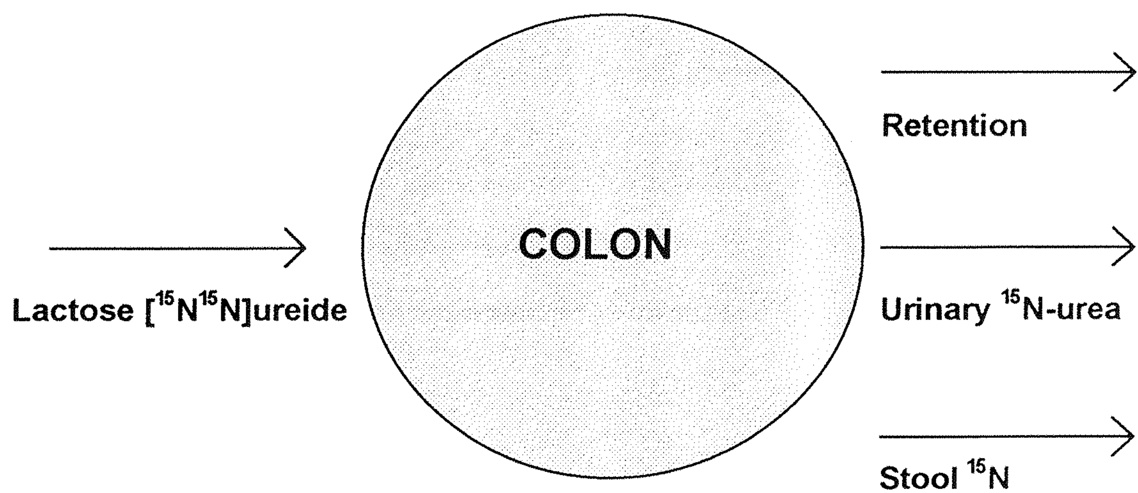
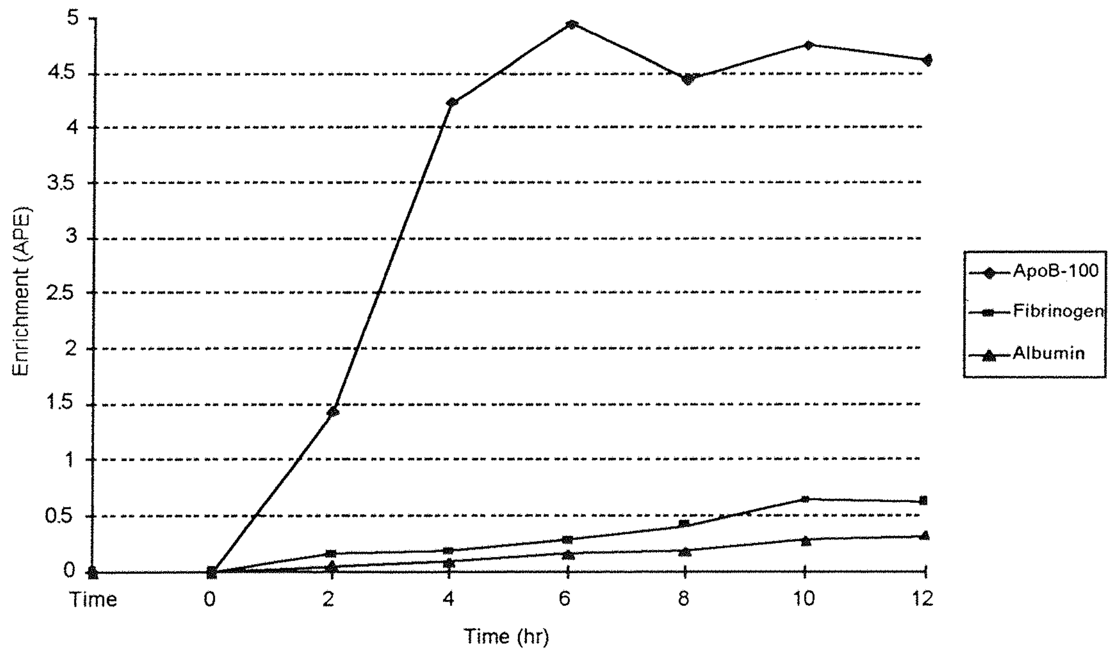


Figure 2.15 Graph to show ^{15}N isotopic enrichment in the lysine fraction of apoB- 100, fibrinogen, and albumin after hourly oral doses of [^{15}N]lysine in one male adult subject.



Appendix 2B Dietary data for subject 1

DAY 1	Portion size	Protein (g)	Energy (J)
BREAKFAST			
Frosted rice cereal	30g	1.3	490
Full-fat milk	200g	6.4	564
English apple juice	200g	0.4	428
LUNCH			
White bread	104g	8.1	1010
Mixed salad (lettuce, tomato, cucumber)	150g	1	55
Potato crisps	30g	1.5	651
Polyunsaturated margarine	20g	0.1	522
Full-fat mayonnaise	40g	0.4	1132
Carbonated beverage	1160ml	0	1949
Banana	131g	1.6	528
DINNER			
Vegetable lasagne	1 unit	13.8	1551
Mixed salad (lettuce, tomato, cucumber)	230g	1.5	84
Full-fat mayonnaise	50g	0.6	1414
Peach and pear fruit cocktail	140g	0.5	421
Double cream	44g	0.8	814
SNACKS			
Fruit sweets	45g	0.3	585
Sponge biscuits	50g	1.8	766
	TOTAL	40.1	12964

DAY 2	Portion size	Protein (g)	Energy (J)
BREAKFAST			
Frosted rice cereal	30g	1.3	490
Full-fat Milk	200g	6.4	564
English apple juice	200g	0.4	428
LUNCH			
White bread	115g	9	1117
Mixed salad (lettuce, tomato, cucumber)	160g	1.1	59
Potato crisps	30g	1.5	651
Polyunsaturated margarine	15g	0.1	392
Full-fat mayonnaise	40g	0.4	1132
Carbonated beverage	330ml	0	554
Banana	250g	3	1008
DINNER			
Potato, cheese, and onion Pasty	2 units	9.8	1902
Mixed salad (lettuce, tomato, cucumber)	250g	1.7	92
Full-fat mayonnaise	50g	0.6	1414
Peach and pear fruit cocktail	140g	0.5	421
Double cream	40ml	0.7	740
SNACKS			
Fruit sweets	60g	0.3	780
Sponge biscuits	50g	1.8	766
	TOTAL	**38.6**	**12510**

DAY 3	Portion size	Protein (g)	Energy (J)
BREAKFAST			
Frosted rice cereal	30g	1.3	490
Full-fat milk	200ml	6.4	564
English apple juice	200ml	0.4	428
LUNCH			
White bread	100g	7.8	971
Mixed salad (lettuce, tomato, cucumber)	150g	1	55
Potato crisps	30g	1.5	651
Polyunsaturated margarine	23g	0.1	600
Full-fat mayonnaise	46g	0.5	1302
Carbonated beverage	660ml	0	1108
Banana	108g	1.3	435
DINNER			
Vegetable provencale	half	7	1885
Potato waffles	2 units	3	902
Mixed salad (lettuce, tomato, cucumber)	230g	1.5	84
Full-fat mayonnaise	45g	0.5	1273
Peach and pear fruit cocktail	140g	0.5	421
Double cream	40ml	0.7	740
SNACKS			
Sponge biscuits	50g	1.8	766
	TOTAL	**35.3**	**12675**

DAY 4	Portion size	Protein (g)	Energy (J)
MEAL 1 - 6.00am			
Frosted rice cereal	30g	1.3	490
Full-fat milk	100g	3.2	282
MEAL 2 - 9.00am			
English Apple Juice	200g	0.4	428
Full-fat milk	100g	3.2	282
Banana	100g	1.2	403
MEAL 3 - 12.00pm			
White Bread	110g	8.6	1068
Mixed salad (lettuce, tomato, cucumber)	160g	1.1	59
Potato crisps	30g	1.5	651
Flora Margarine	18g	0.1	470
Full-fat mayonnaise	50g	0.6	1414
Carbonated beverage	330ml	0	554
MEAL 4 - 3.00pm			
Banana	100g	1.2	403
Carbonated beverage	330ml	0	554
Sponge biscuits	25g	0.9	383
MEAL 5 - 6.00pm			
Potato, cheese, and onion pasty	2 units	9.8	1902
Mixed salad (lettuce, tomato, cucumber)	250g	1.7	92
Full-fat mayonnaise	50g	0.6	1414
MEAL 6 - 9.00pm			
Peach and pear fruit cocktail	140g	0.5	421
Double cream	40ml	0.7	740
Sponge biscuits	25g	0.9	383
SNACKS			
Fruit sweets	45g	0.3	585
	TOTAL	**37.7**	**12979**

DAY 5	Portion size	Protein (g)	Energy (J)
BREAKFAST			
Frosted rice cereal	30g	1.3	490
Full-fat milk	200g	6.4	564
English apple juice	200g	0.4	428
LUNCH			
White bread	108g	8.4	1049
Mixed salad (lettuce, tomato, cucumber)	160g	1.1	59
Potato crisps	30g	1.5	651
Polyunsaturated margarine	23g	0.1	600
Full-fat mayonnaise	35g	0.4	990
Carbonated beverage	330ml	0	554
Banana	108g	1.3	435
DINNER			
Vegetable provencale	half	7	1885
Potato waffles	2 units	3	902
Full-fat mayonnaise	20g	0.2	553
Peach and pear fruit cocktail	140g	0.5	421
Double cream	40ml	0.7	740
SNACKS			
Sponge biscuits	50g	1.8	766
Lager beer	880ml	1.8	1056
	TOTAL	**35.9**	**12143**

AVERAGE PROTEIN INTAKE	***37.5 ± 2.0g/d***
AVERAGE ENERGY INTAKE	***12654 ± 348kJ/d***

Appendix 2C Reagents for assays

Alkaline Hypochlorite

Stock: 25 g sodium hydroxide in 800 ml deionised water plus 40 ml 5 g/100 g NaOCl solution, made up to one litre.

Working Solution:100 ml stock solution added to 400 ml deionised water.

PNP Solutions

Stock: 50 g phenol in 500 ml deionised water plus 250 mg sodium nitroprusside in 100 ml deionised water (dissolved separately) made up to one litre.

Working Solution:100 ml stock solution added to 300 ml deionised water

Urease Solutions

Stock: 200 mg Jack Bean urease type III in 100 ml deionised water and 100 ml glycerol

Working Solution: 1 ml stock urease made up to 100 ml with EDTA buffer (10 g Na_2 - EDTA/100 ml deionised water, adjusted to pH 6.5)

Urea Standards

Standards of 2.5, 5.0, 7.5 and 10.0 µl urea-N/25 µl were made from a solution of 500 mg urea-N/100 ml deionised water containing 100 mg sodium azide.

Citrate Buffer

21.03 g of citric acid and 8 g of sodium hydroxide were added to 800 ml of deionised water. pH was adjusted to 3.41 with concentrated hydrochloric acid solution and the volume made up to one litre. Buffer was made fresh each day.

Enyme Inhibitor

2g Soybean trypsin inhibitor

1g Thimerosal

2g Sodium azide

10g Na_4 - EDTA

made up in 100ml deionised water and stored at -70°C until required.

Lithium Hypobromite Solution

1 ml bromine solution added to 30 ml 10g/100g lithium hydroxide solution

Isomolar NaCl / EDTA solution

0.1 M Na_4 - EDTA

0.04 M Sodium chloride

made up in deionised water and stored at 4°C until required.

Saline EDTA solution

0.195 M Sodium chloride

1 mM Na_4 - EDTA

Adjusted to pH 7.4: d = 1.006 g/ml

CHAPTER 3

Metabolism of lactose [^{13}C]ureide

3.1 Introduction

This chapter describes the metabolism of a single oral dose of lactose [^{13}C]ureide, which was administered to nine adult subjects consuming a diet marginally adequate in protein.

A model for the proposed metabolism of lactose [^{13}C]ureide is detailed in section 2.5.1. It is assumed that a single oral dose of lactose [^{13}C]ureide will traverse the upper gut intact. Once it reaches the colon, it will be fermented and hydrolysed by the resident microflora and release ^{13}C-labelled carbon dioxide. $^{13}CO_2$ is absorbed across the colonic mucosa and excreted in the breath. By observing the total excretion of breath $^{13}CO_2$ over time, a direct measure of lactose [^{13}C]ureide fermentation and hydrolysis can be obtained. The aim of the study was to determine whether lactose-ureide is a suitable vehicle to *non-invasively* deliver a *known dose* of isotopic labelled urea *directly* to the colon.

In the quest to understand body nitrogen metabolism and nitrogen requirements, urea kinetics methodology has been widely used to study body urea dynamics. Isotopically labelled [$^{15}N^{15}N$]urea is introduced into the body, normally orally or intravenous, and label excreted into the urine and stool is determined experimentally. From this data, urea hydrolysed in the colon, and the urea-N salvaged and subsequently retained within the body nitrogen pool, can be calculated. The degree of nitrogen retention which has been observed under different metabolic circumstances has highlighted the important contribution of salvaged urea-N to overall nitrogen homeostasis.

In the calculation of urea kinetics, the value for the salvage of urea-N in the body is derived by the difference between the rate of urea production and excretion. Salvaged urea-N which is retained in the body is subsequently derived from the difference between the values for salvage and urea recycling. Potential errors may be introduced at both these stages of calculation, and so it is unclear whether retention of urea-N can be accurately quantified using urea kinetics methodology. In order to quantify the extent of urea-N retention, a known quantity of label must be presented to the site of bacterial hydrolysis directly. This has been achieved previously by utilising the biopsy channel of a colonoscope (Moran and Jackson, 1991*a*), but this invasive method is not widely applicable to study normal individuals. Lactose-ureide, isotopically labelled on the urea part of the molecule, may provide a means of non-invasively introducing a known dose of ^{15}N to individuals which will pass to the colon intact.

Lactose-ureide is formed from the covalent bonding of urea to lactose. The characteristics of lactose-ureide, and other glycosyl-ureides, were first described by Schoorl in 1903. Later work in animals demonstrated that the bond between lactose and urea is resistant to cleavage in the upper gastrointestinal tract, but may be hydrolysed by the actions of the colonic microflora (Hofmann, 1931; Merry *et al*, 1982*a,b*). Heine and colleagues (1995) have recently used lactose [^{13}C]ureide to estimate gastrointestinal motility in normal adults. The time taken for breath to become significantly enriched with $^{13}CO_2$ is taken to represent the time taken for lactose [^{13}C]ureide to pass from the mouth to the site of bacterial hydrolysis in the colon. This is then defined as

the orocoecal transit time for material from the oral cavity to the entrance of the colon.

This chapter describes a study of lactose [^{13}C]ureide metabolism in nine adults who had adapted to a diet low in protein, to assess the effectiveness of lactose-ureide for the purposes of the thesis.

3.2 Methods

A clinical trial was set up. Nine healthy adult volunteers agreed to participate after the nature of the study had been explained to them. Sex, age, and BMI of the subjects are summarised in section 2.3.1. Subjects consumed a diet marginally adequate in protein for five days (section 2.3.2). All subjects were instructed to fast from 6.00 pm on day three. At 6.00 am on day four, subjects received a single oral dose of 500 mg lactose [^{13}C]ureide dissolved in deionised water. Lactose [^{13}C]ureide had been previously synthesised and analysed for purity (section 2.2). Subjects breakfasted immediately after administration of the dose, and were then given meals at 3 hourly intervals as described in section 2.3.2. Carbon dioxide excretion was measured for 10 min in every hour from 6.00 am to 11.00 pm using indirect calorimetry. Measurements were made while the subjects lay at rest, and they were asked to restrict their activity as much as possible between measurements. A sample of breath was collected into a sealed breath bag every hour from 6.00 am to 11.00 pm, and then at time points 24, 30, 36 and 48 hr after administration of dose. Breath samples were analysed for $^{13}CO_2$ enrichment by isotope-ratio mass-spectrometry (section 2.4.2).

3.3 Results

3.3.1 Diets

Values for protein and energy consumed by subjects are shown in table 3.1, expressed as a daily mean over the five day study period.

Table 3.1 Mean intakes of protein and energy over five days for nine subjects consuming a diet marginally adequate in protein

Subject	*Protein intake (SD) g/d*	*Energy intake (SD) MJ/d*
1	37.5 (2.0)	12.65 (0.35)
2	35.5 (2.1)	10.70 (0.12)
3	32.5 (1.0)	7.98 (0.28)
4	34.3 (2.2)	9.37 (0.89)
5	36.4 (1.8)	11.16 (0.13)
6	36.6 (2.4)	11.86 (0.12)
7	38.6 (1.9)	13.94 (0.20)
8	35.3 (2.1)	9.63 (0.22)
9	36.9 (2.5)	11.65 (0.42)

On average, subjects consumed 36.0 (SD 1.8) g protein and 11.00 (SD 1.81) MJ energy each day.

3.3.2 Breath $^{13}CO_2$ excretion

Individual results for the profile of $^{13}CO_2$ excretion on the breath over time, expressed as a percentage of dose given, are shown in figures 3.1 to 3.9 (Appendix 3A). Results were calculated as described in section 2.5.3. Little $^{13}CO_2$ was excreted during the first 6 h after administration of the dose, followed by a sharp rise in excretion up to 17 h in most subjects. For all subjects, measured levels of breath $^{13}CO_2$ enrichment reached a maximum between 9 and 16 h after ingestion of the dose, an average of 13.6 (SD 2.9) h. Excretion of label at time points after 17 hr were calculated using the CO_2 production rate

value measured at the same time on the previous day. Breath enrichment tended to background levels of ^{13}C isotopic abundance by about 48 hr in all subjects.

Individual results for the cumulative excretion of breath $^{13}CO_2$ expressed as a percentage of the dose given are show in figures 3.10 to 3.18 (Appendix 3A). Less than 5 % of the total dose of lactose [^{13}C]ureide was excreted before 6 h in most subjects. All subjects tended toward isotopic plateau after 48 h.
On average, 5.2 (SD 0.8) % of the total dose was excreted 6 h after administration. Mean cumulative excretion of ^{13}C on breath was 45.3 (SD 13.6) % - range 14.6 to 60.6 % - of the dose after 17 hr (Figure 3.19). This rose to 79.4 (SD 24.3) % - range 40.5 to 122.4 % - of the dose after 48 hr (Figure 3.20).

On average, 57 % of the total dose excreted after 48 hr was excreted by 17 h. Mean cumulative excretions of $^{13}CO_2$ on breath excreted after 17 and 48 h expressed as a percentage of dose are shown in table 3.2

Table 3.2 Cumulative excretion of $^{13}CO_2$ on breath in nine adults consuming a diet marginally adequate in protein following a single oral dose of lactose [^{13}C]ureide

Subject	*% dose excreted after 17 h*	*% dose excreted after 48 h*
1	44.3	83.1
2	37.9	82.4
3	46.9	59.2
4	53.0	66.6
5	14.6	40.5
6	58.9	122.4
7	60.6	107.0
8	47.3	72.0
9	44.1	76.8
MEAN (SD)	45.3 (13.6)	79.4 (24.3)

3.4 Discussion

Subjects consumed all the food given to them over the entire study period with only one exception. Most subjects consumed over 35 g/protein/d, as an average over 5 days, which has been shown to be adequate in maintaining apparent nitrogen balance in normal adult men (Langran *et al*, 1992). Subjects 3 and 4 failed to attain this intake of protein. However, no subject consumed 30 g/protein/d or less, where previously nitrogen equilibrium was not maintained in six adult men (Danielsen and Jackson, 1992).

Orocoecal transit time, the time taken for food to pass from the oral cavity to the entrance of the colon, has been reported at between 4.3 and 9 hr (Seeley *et al*, 1988), depending on whether the ingested matter is of a liquid or solid nature respectively. $^{13}CO_2$ was detected in the breath of all subjects (except subject 1) after 3 h with the highest value in subject 9 (calculated as 2% of the total dose administered). In another study, $^{13}CO_2$ was not detected before 6 h after 1g lactose [^{13}C]ureide was given orally in one adult subject (Heine *et al*, 1995). There are a number of possible reasons to explain the variability and early detection of label in the present study. It is not likely that there was variation in background excretion of $^{13}CO_2$ between individuals, since an increase in enrichment was detected in 8 of the 9 subjects. This consistent rise may indicate that the meal consumed by subjects at 6.00 am contained some food with high natural ^{13}C abundance, possibly cane sugar. Alternatively, early detection of label may represent an amount of lactose [^{13}C]ureide hydrolysed in the upper gastrointestinal tract, although over 99.9 % of gastrointestinal bacteria are located in the colon, and only a very small number in the terminal ileum, of normal adults (Gustafsson, 1982). It is also possible that orocoecal transit time for some material is more swift than that reported by Heine and colleagues. Indeed, Wutzke *et al* (1997) have recently reported detecting $^{13}CO_2$ on the breath after 1.5 h in 3 healthy adults after a single oral dose of 1g lactose [^{13}C, ^{15}N]ureide, and have calculated average orocoecal transit time in 12 normal adults as 3.0 (SD 1.4) h.

After 6 h, a rapid increase in breath $^{13}CO_2$ enrichment was detected in all subjects, except subject 5. Maximum measured excretion of label varied from 9 to 16 h and is consistent with that reported by Heine *et al* (1995) in one normal adult (8 h) and Wutzke *et al* (1997) in 12 healthy adults (mean of 10 h). Breath $^{13}CO_2$ excretion rate decreased over the final period of the study, although there was a second increase in some subjects after 24 h (most notably subjects 1 and 7).

Comparing cumulative excretion of $^{13}CO_2$ after a single oral dose of lactose [^{13}C]ureide to other reported data, the mean value in the present study of 62.3 (SD 17.6) % of the dose after 24 h is high. Heine *et al* (1995) observed an average excretion of 37.1 (SD 8.1) % as breath $^{13}CO_2$ after 28 h in 14 normal adults, similar to the value an average of 35.2 % after 24 h in 12 healthy adults, reported by Wutzke *et al* (1997). One obvious difference is that the diet given to subjects in the present study was marginally adequate in protein, compared with the standard mixed meals reported in the other two studies. Therefore, the increase in total excretion of breath $^{13}CO_2$, 24 h after a single dose of lactose [^{13}C]ureide, could be as a result of increased bacterial activity in the colon of subjects consuming the lower protein diet. It is also possible that differences in the indigenous diet between subject groups has caused differences in the bacterial population characteristics within the colon, resulting in a different inherent capacity for the fermentation of lactose-ureide and hydrolysis of urea. Wutzke and colleagues (personal communication) have recently characterised human aerobic colonic bacteria which will release $^{13}CO_2$ from lactose [^{13}C]ureide, and these include *Streptococcus sp., Staphlyococcus aureus, Pseudomonas aeroginosa* and *Proteus sp.* among the most active.

In the present study, a further rise in $^{13}CO_2$ enrichment was observed in some subjects (most notably subjects 1 and 7) after 24 h, resulting in a secondary measured peak at 30 h after administration of dose. This is taken to represent absorbed label which has been delayed in a compartment of the body bicarbonate pool before excretion in the breath. The two other studies observing lactose [^{13}C]ureide metabolism take no account of the release of any 'trapped' $^{13}CO_2$ after about 24 h, since measurements were not taken beyond this time. In

the present study, a slow continual excretion of $^{13}CO_2$ in breath between about 36 and 48 h was observed in most subjects, and may represent 'trapped' label released from slowly turning over bicarbonate compartments.

The calculated cumulative excretion of breath $^{13}CO_2$ after 48 h exceeded 100% in subjects 6 and 7, and these results need explanation. One likely possibility which would lead to an overestimate in cumulative breath $^{13}CO_2$ excretion is the effect of foods containing a high natural abundance of ^{13}C consumed in the study diet. Cane sugar has high natural ^{13}C enrichment, and may have been present in the frosted rice cereal given to subjects at 6.00 am, and in the carbonated beverages consumed at 12.00 pm and 3.00 pm. This would lead to an overestimate for breath $^{13}CO_2$ excretion in all subjects, an error which would be cumulative over the 48 h period. Another possibility is that a degree of measurement error may have been introduced during the calculation of cumulative $^{13}CO_2$ excretion. During the study, $^{13}CO_2$ excretion rates for time points at 24, 30, 36 and 48 h were estimated from results obtained at similar times on the previous day. Although these values represent the best estimate available, any deviation from the true value will affect the results. Specifically, if the estimate of CO_2 production used at any particular time point was higher than the true value, this would lead to an overestimate in cumulative $^{13}CO_2$ excretion in breath. Another point to consider concerning measurements is that the cumulative excretion of breath $^{13}CO_2$ after 17 hours following the administration of dose was taken at 6 to 12 hourly intervals (24, 30, 36 and 48 h). Any changes in breath $^{13}CO_2$ enrichment, or in total CO_2 excretion, between these time points were not directly measured. These errors may be additive, and lead to an overestimation of cumulative $^{13}CO_2$ excretion on breath. As well as these variations, there is also likely to be an inherent variation in $^{13}CO_2$ excretion between subjects, based upon their individual capacity to ferment, hydrolyse, and excrete label. The range of apparent cumulative $^{13}CO_2$ excretion on breath was 40.5 to 122.4 % over the 9 subjects. Subjects 6 and 7 would appear to display the most efficient metabolism of lactose [^{13}C]ureide of all the subjects. The fact that apparent excretion exceeded 100 % in both subjects may be indicative of the additional effect of ^{13}C enriched foods consumed in the diet on

total breath $^{13}CO_2$ excretion, and limitations of the measurement and subsequent calculation of cumulative $^{13}CO_2$ excretion

In summary, this chapter has demonstrated that a single oral dose of lactose [^{13}C]ureide is resistant to metabolism in the upper gastrointestinal tract. Once it reaches the colon, it is rapidly hydrolysed, and most of the label is excreted as breath $^{13}CO_2$ over 48 h, although there is wide individual variation. Therefore, it is concluded that lactose-ureide is a suitable way to *non-invasively* transport a *known dose* of isotopic label *directly* to the colon. Labelled with ^{15}N on the urea part, it offers a potential means of quantifying the extent of urea-N salvage in the colon, and the subsequent retention of nitrogen in the body.

Appendix 3A Figures

Figure 3.1 Excretion profile of $^{13}CO_2$ in breath following a single oral dose of lactose [^{13}C]ureide in subject 1

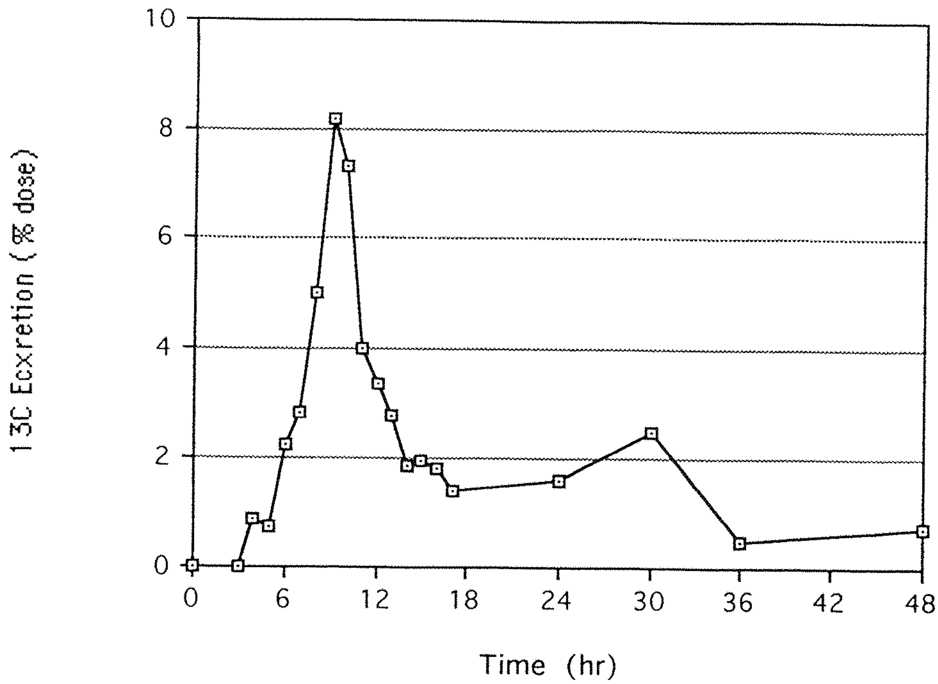


Figure 3.2 Excretion profile of $^{13}CO_2$ in breath following a single oral dose of lactose [^{13}C]ureide in subject 2

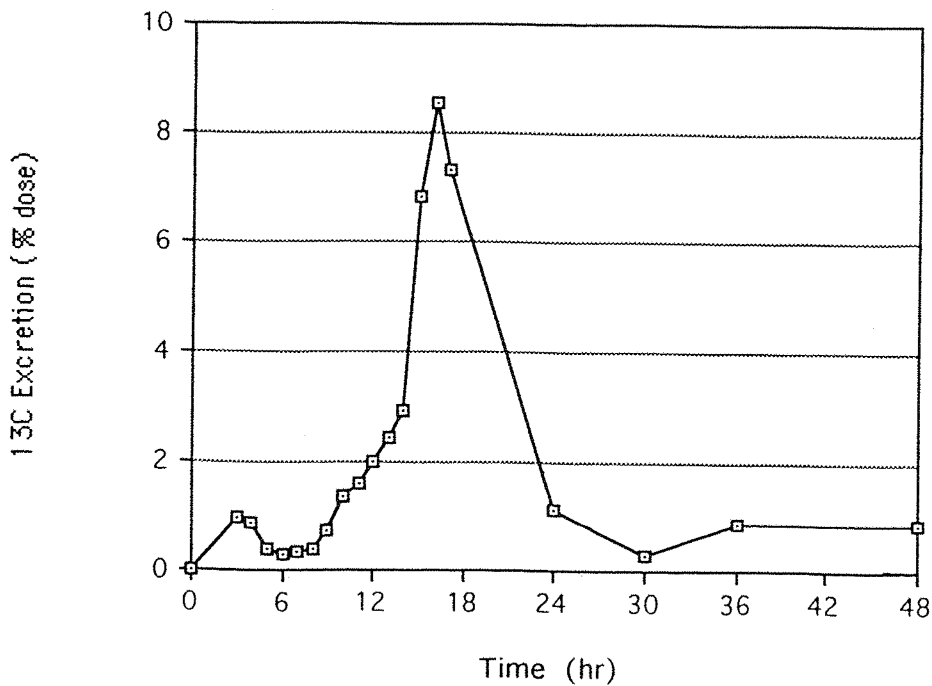


Figure 3.3 Excretion profile of $^{13}CO_2$ in breath following a single oral dose of lactose [^{13}C]ureide in subject 3

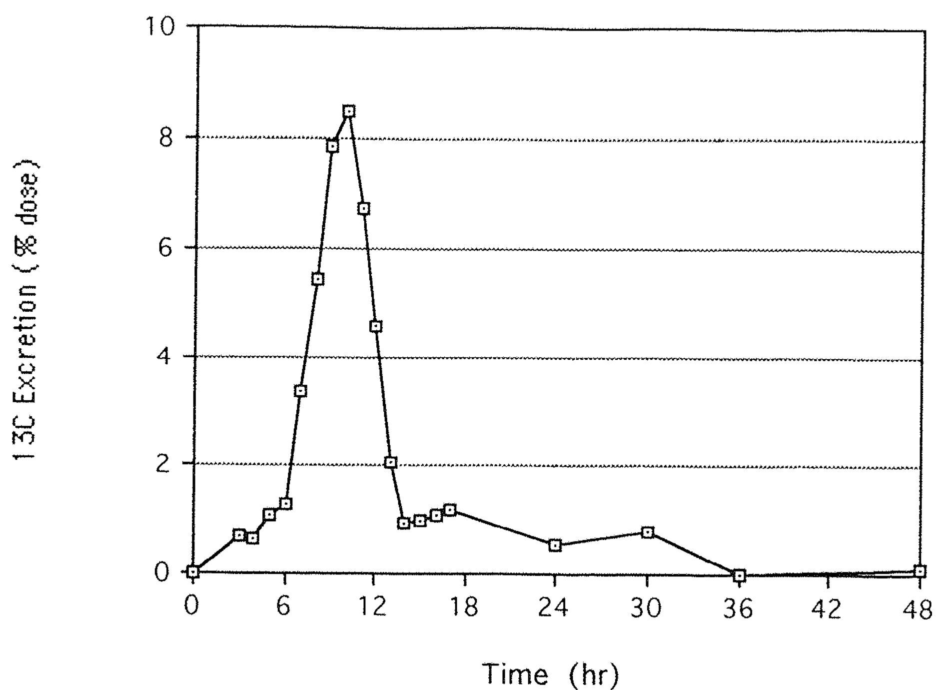


Figure 3.4 Excretion profile of $^{13}CO_2$ in breath following a single oral dose of lactose [^{13}C]ureide in subject 4

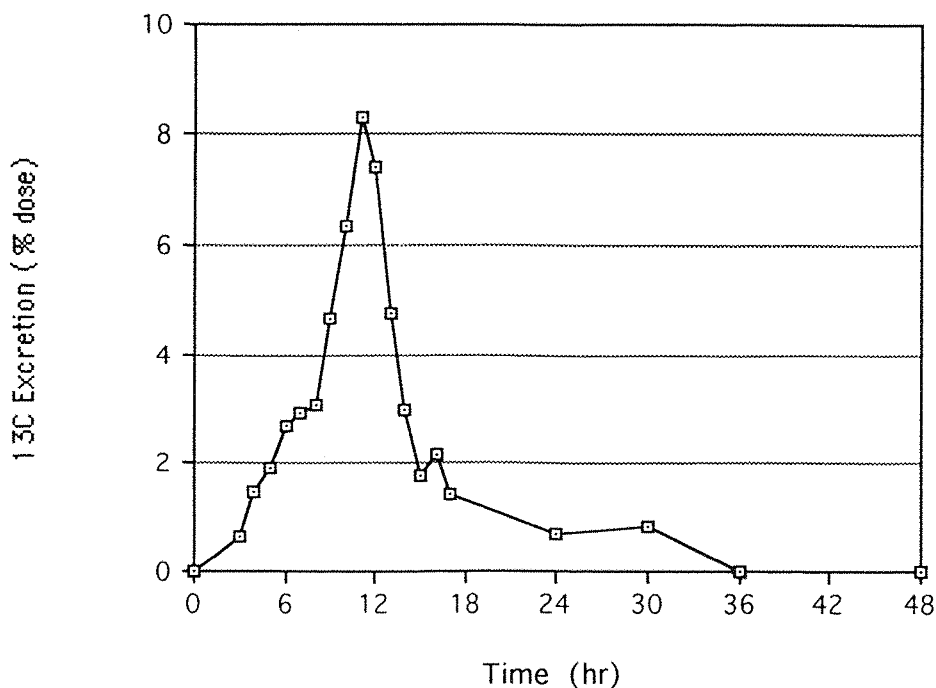


Figure 3.5 Excretion profile of $^{13}CO_2$ in breath following a single oral dose of lactose [^{13}C]ureide in subject 5

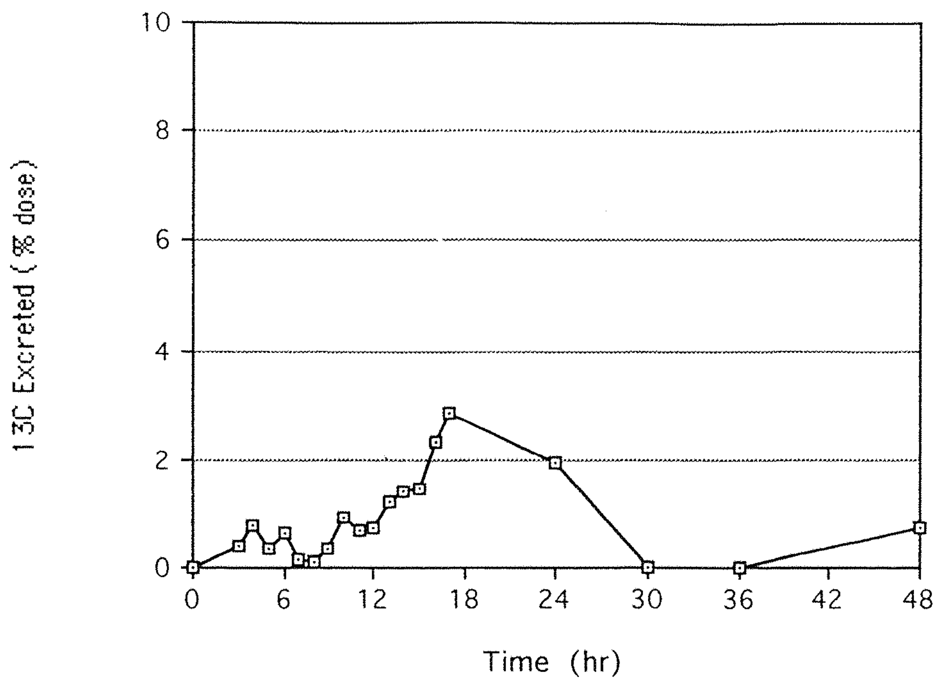


Figure 3.6 Excretion profile of $^{13}CO_2$ in breath following a single oral dose of lactose [^{13}C]ureide in subject 6

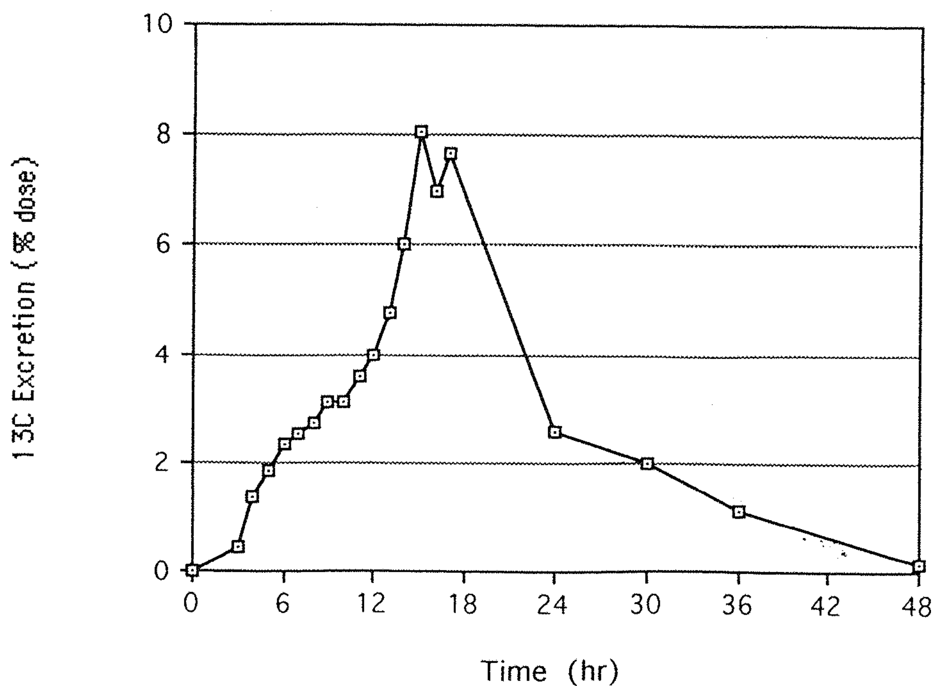


Figure 3.7 Excretion profile of $^{13}CO_2$ in breath following a single oral dose of lactose [^{13}C]ureide in subject 7

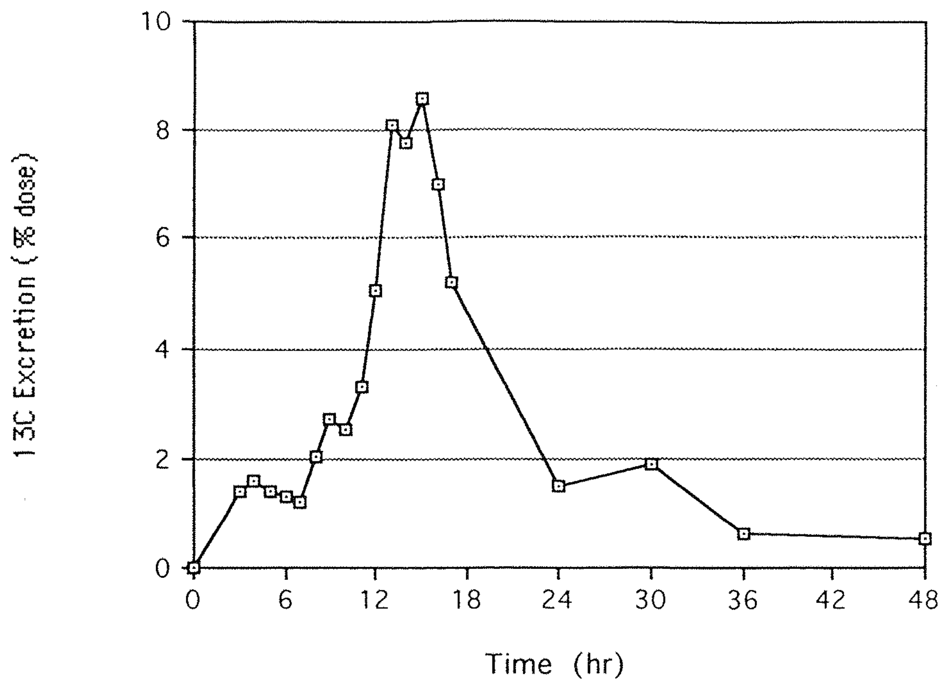


Figure 3.8 Excretion profile of $^{13}CO_2$ in breath following a single oral dose of lactose [^{13}C]ureide in subject 8

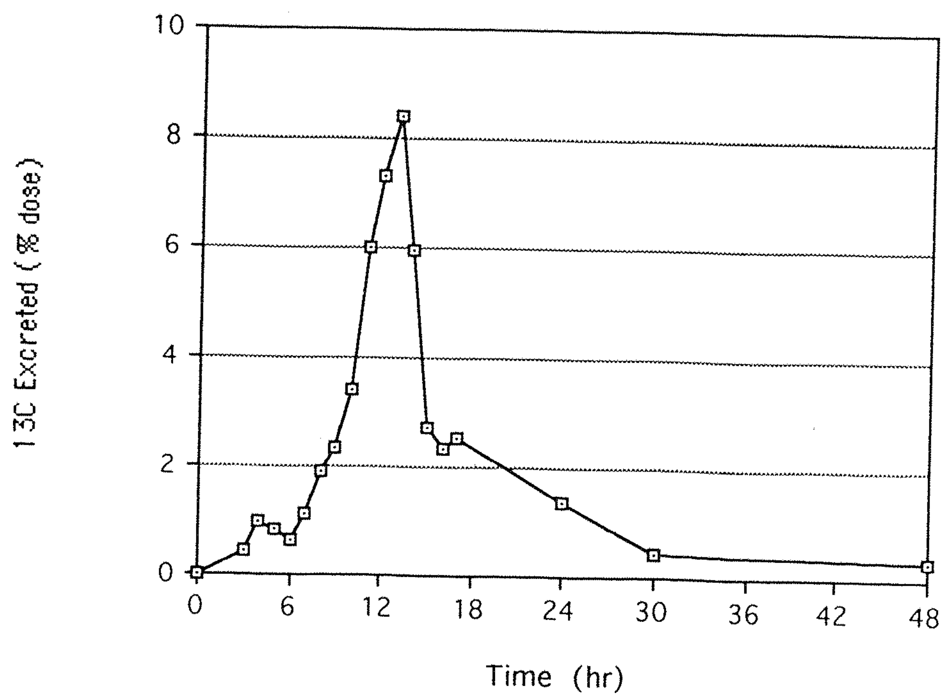


Figure 3.9 Excretion profile of $^{13}CO_2$ in breath following a single oral dose of lactose [^{13}C]ureide in subject 9

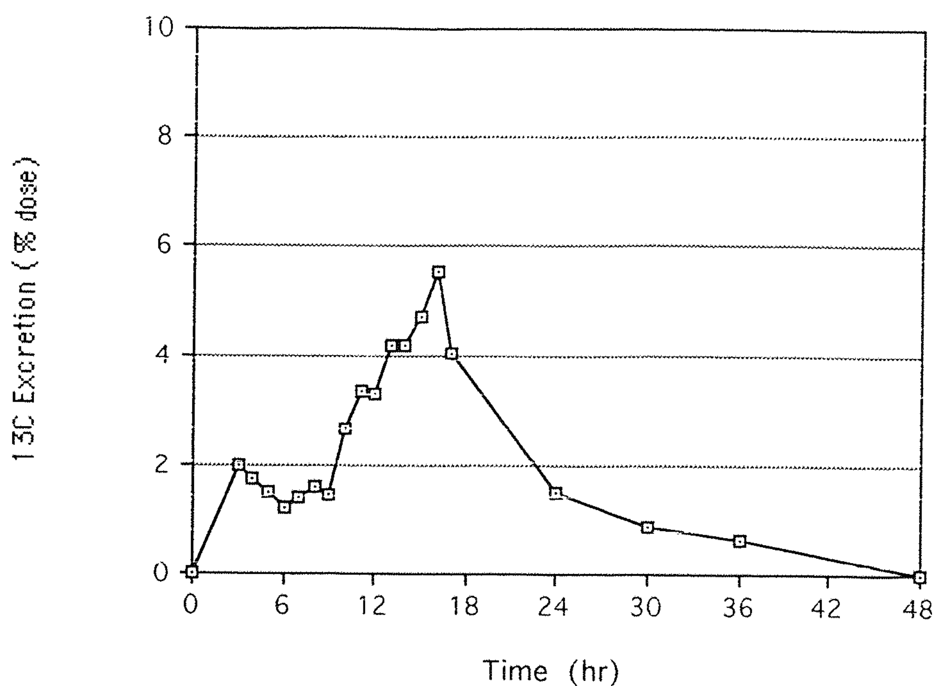


Figure 3.10 Cumulative excretion of $^{13}CO_2$ in breath following a single oral dose of lactose [^{13}C]ureide in subject 1

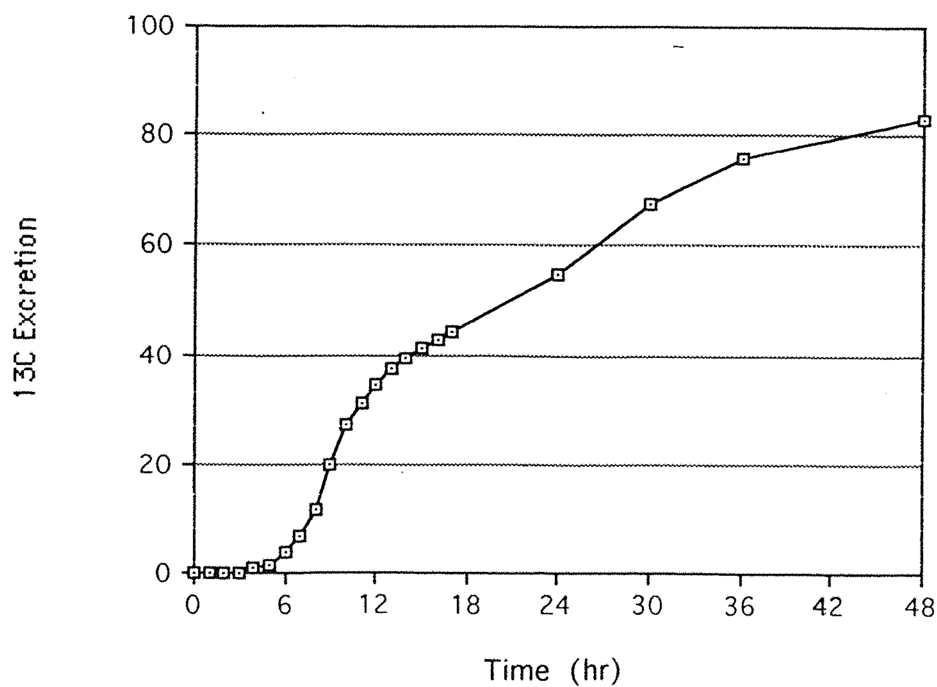


Figure 3.11 Cumulative excretion of $^{13}CO_2$ in breath following a single oral dose of lactose [^{13}C]ureide in subject 2

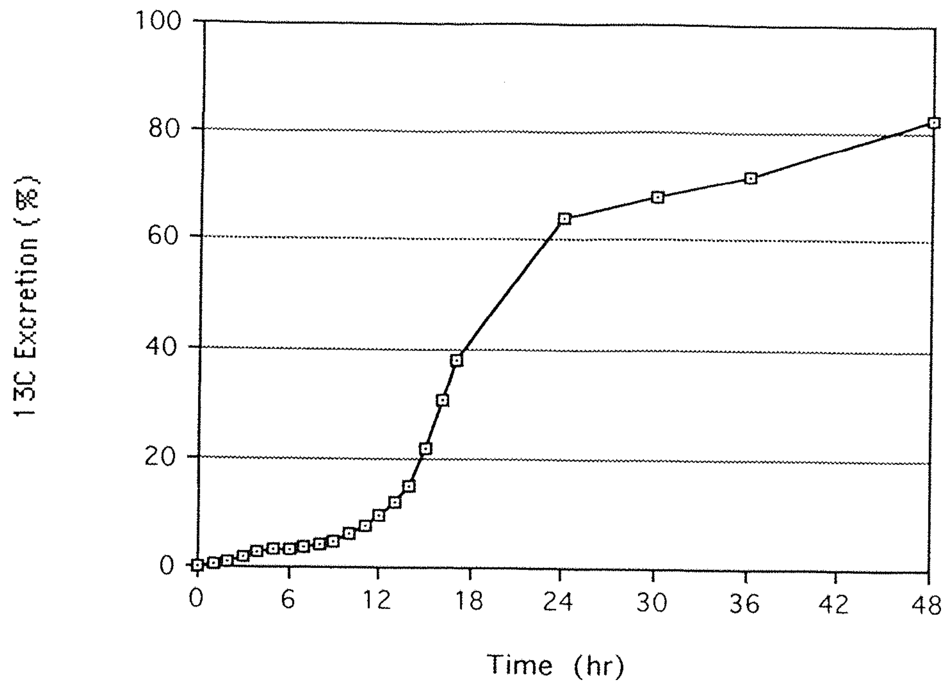


Figure 3.12 Cumulative excretion of $^{13}CO_2$ in breath following a single oral dose of lactose [^{13}C]ureide in subject 3

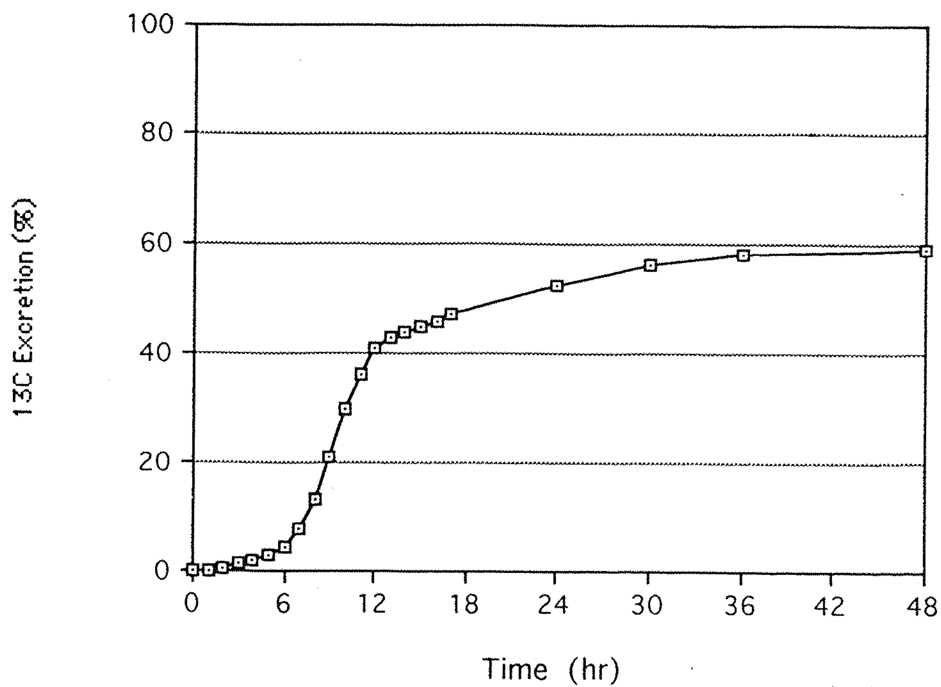


Figure 3.13 Cumulative excretion of $^{13}CO_2$ in breath following a single oral dose of lactose [^{13}C]ureide in subject 4

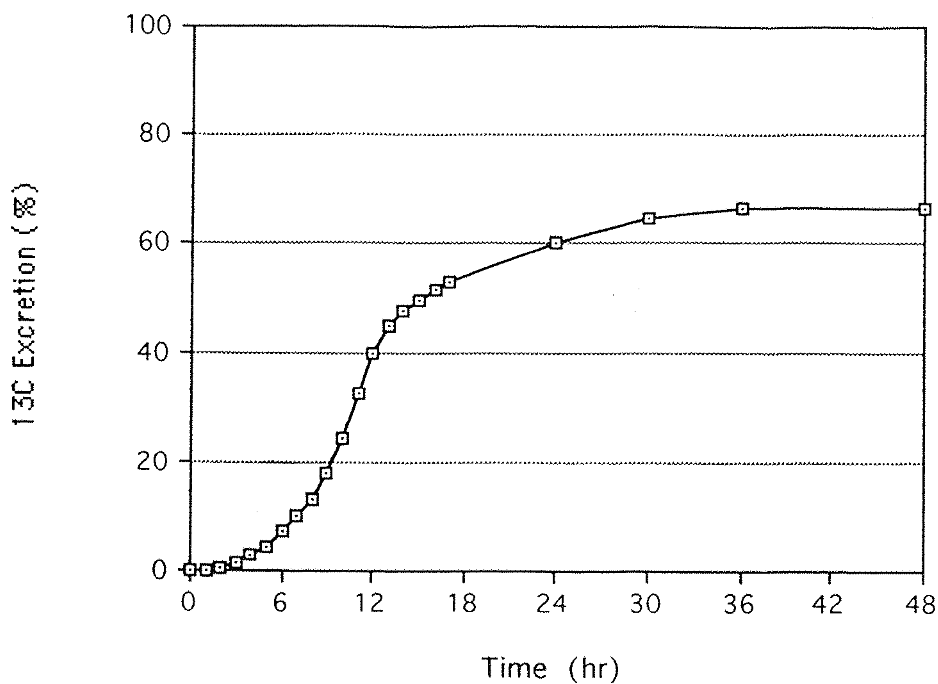


Figure 3.14 Cumulative excretion of $^{13}CO_2$ in breath following a single oral dose of lactose [^{13}C]ureide in subject 5

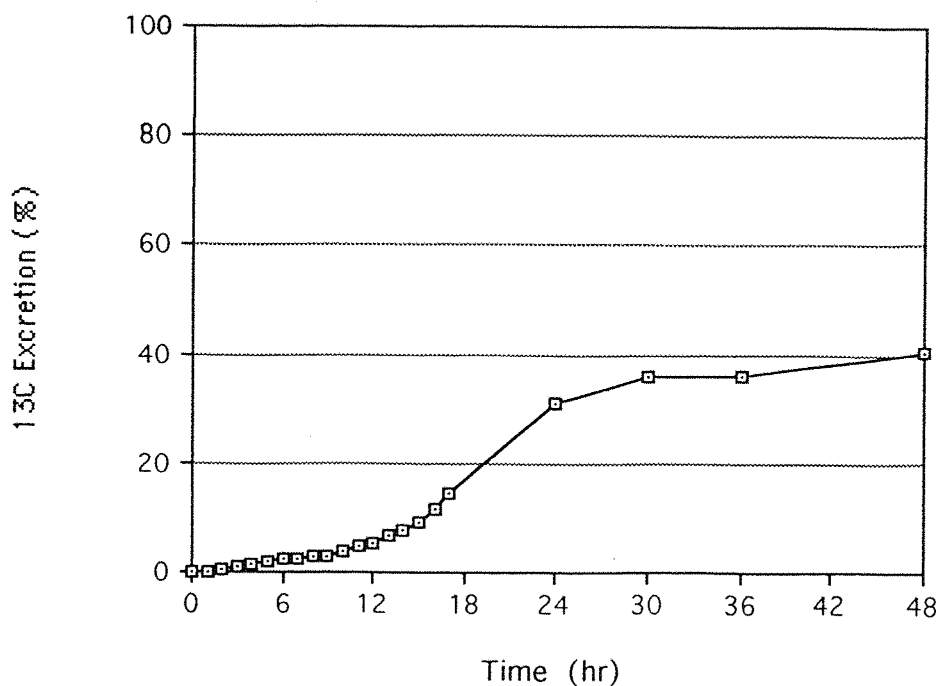


Figure 3.15 Cumulative excretion of $^{13}CO_2$ in breath following a single oral dose of lactose [^{13}C]ureide in subject 6

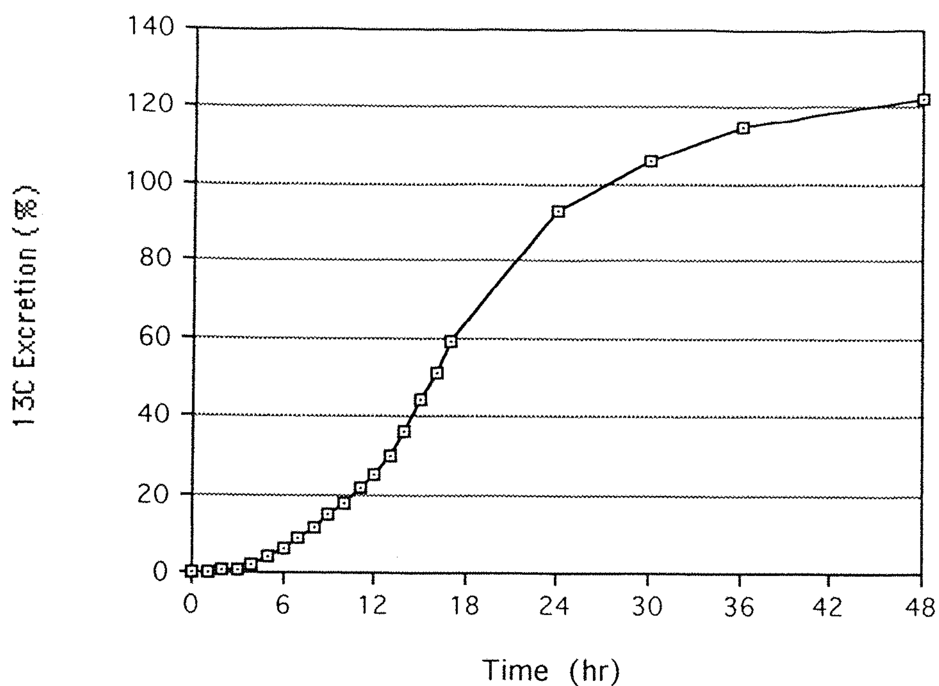


Figure 3.16 Cumulative excretion of $^{13}CO_2$ in breath following a single oral dose of lactose [^{13}C]ureide in subject 7

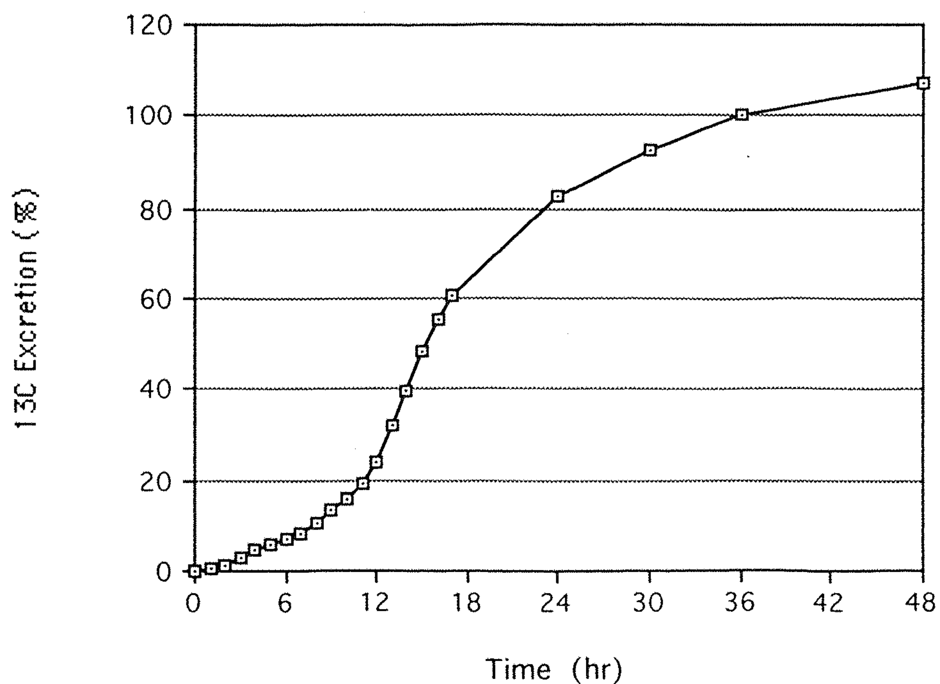


Figure 3.17 Cumulative excretion of $^{13}CO_2$ in breath following a single oral dose of lactose [^{13}C]ureide in subject 8

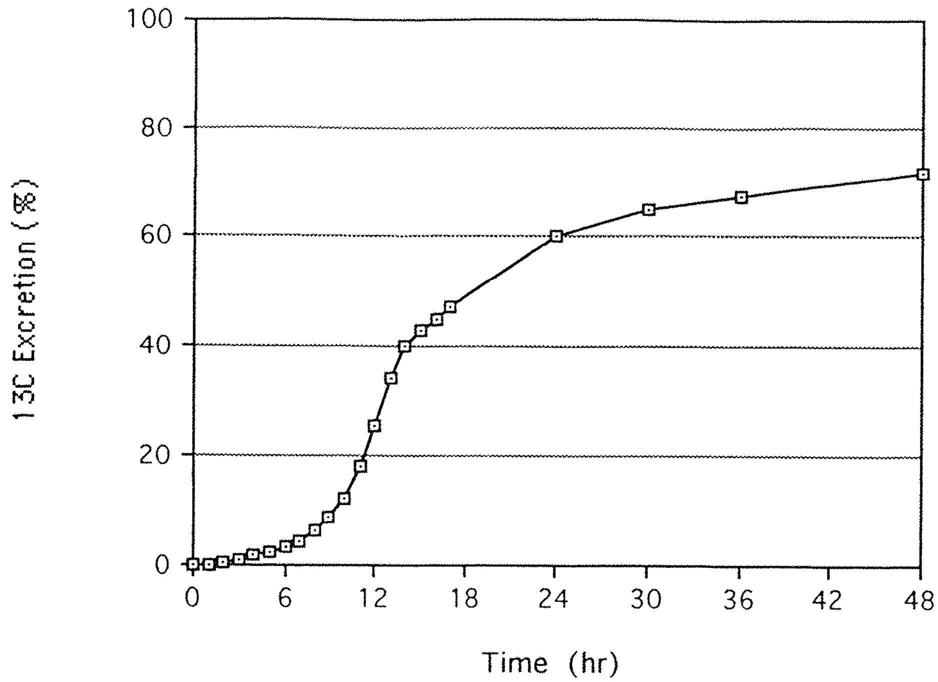


Figure 3.18 Cumulative excretion of $^{13}CO_2$ in breath following a single oral dose of lactose [^{13}C]ureide in subject 9

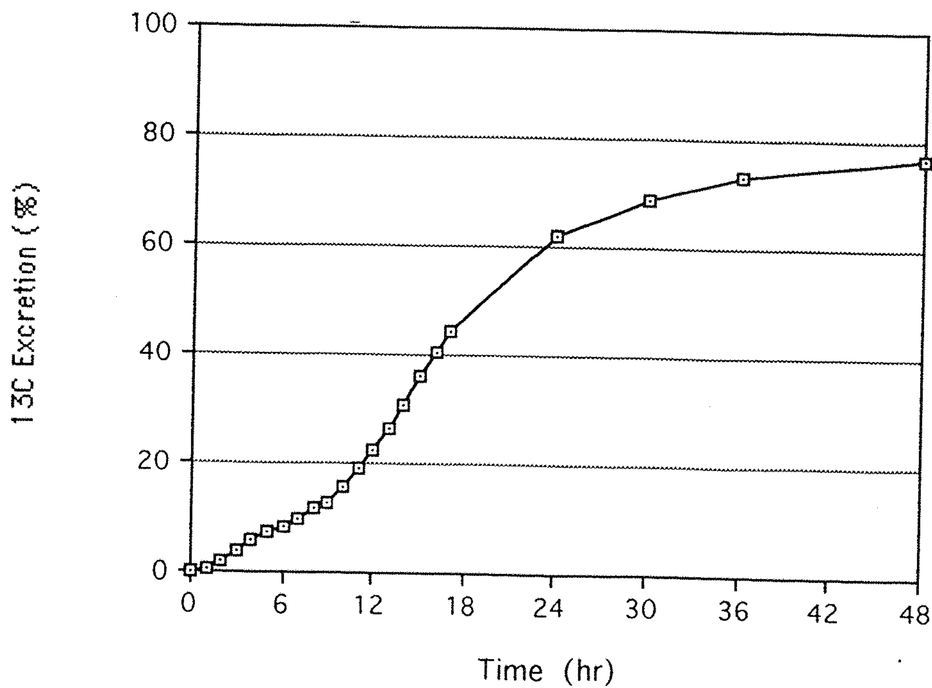


Figure 3.19 Cumulative excretion of breath $^{13}CO_2$ in nine adults consuming a diet marginally adequate in protein over 17 h following a single oral dose of lactose [13C]ureide. Values expressed as mean ± SD

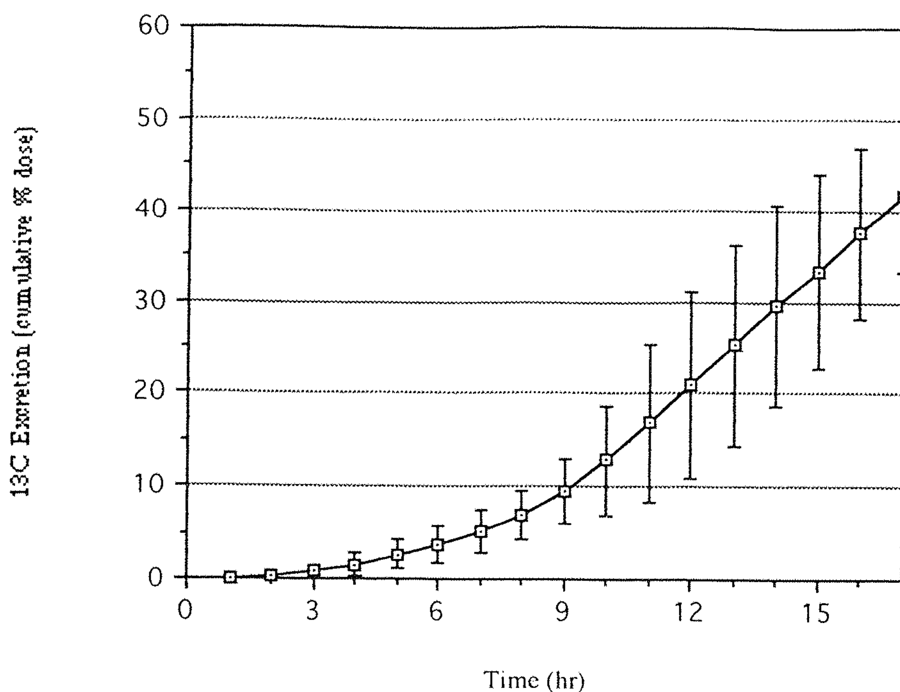
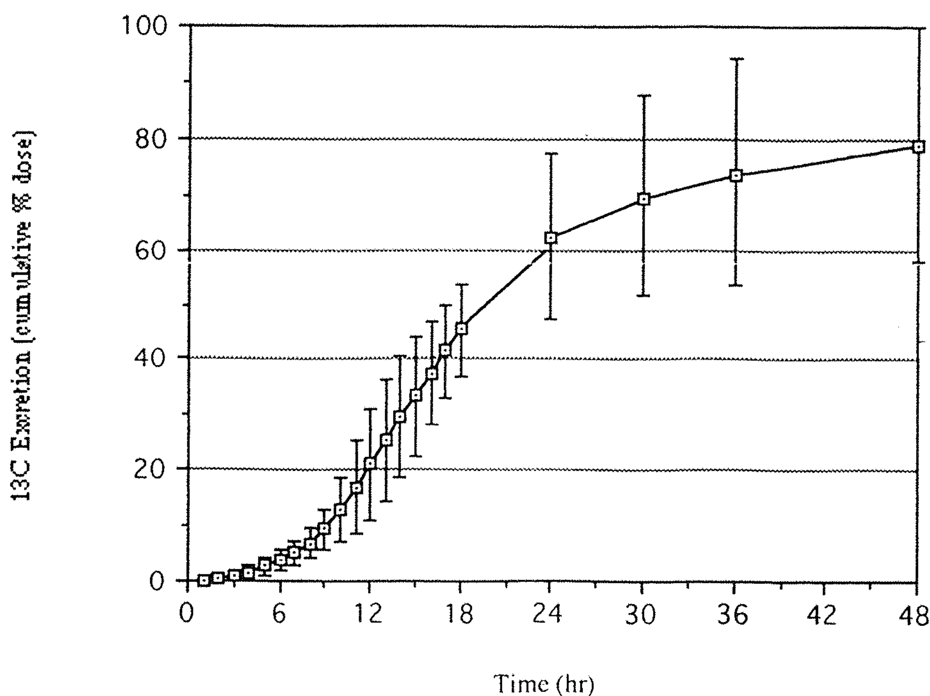


Figure 3.20 Cumulative excretion of breath $^{13}CO_2$ in nine adults consuming a diet marginally adequate in protein over 48 h following a single oral dose of lactose [13C]ureide. Values expressed as mean ± SD



CHAPTER 4

Metabolism of lactose [$^{15}N^{15}N$]ureide

4.1 Introduction

This chapter details the metabolism of a prime and intermittent oral doses of lactose [$^{15}N^{15}N$]ureide, which were administered to nine adult subjects consuming a diet marginally adequate in protein.

A model for the proposed metabolism of lactose [$^{15}N^{15}N$]ureide is detailed in section 2.5.4. As with lactose [^{13}C]ureide, the labelled compound is presumed to traverse the upper gut intact. Once it reaches the colon, it is fermented by the colonic microflora to release lactose and [$^{15}N^{15}N$]urea. The urea may then be further hydrolysed to release $^{15}NH_3$.

The previous chapter demonstrated that most of a single oral dose of lactose [^{13}C]ureide was fermented and hydrolysed in the colon of nine subjects consuming a diet marginally adequate in protein. This suggests that lactose-ureide is a useful vehicle to non-invasively deliver a known quantity of isotope directly to the colon.

The next step was to orally administer lactose [^{15}N^{15}N]ureide to the subjects under the same study conditions. The aim of the study was to determine the extent of the movement of ^{15}N from lactose [^{15}N^{15}N]ureide into *urinary urea* and *stool.* From these measurements, the metabolic fate of nitrogen from [^{15}N^{15}N]urea salvaged in the colon, and subsequently retained in the body nitrogen pool, can be determined.

In this study, it is important to be clear about *what* was measured, and *how* it was measured. In urea kinetics methodology, [^{15}N^{15}N]urea can been administered to subjects either as a *single* dose, or as *intermittent* doses. The single dose method works on the principal of *amounts.* A single dose of a known amount of [^{15}N^{15}N]urea is administered, usually orally, and the total amount of label exiting the body as urinary urea is used to calculate the salvage and retention of label as a proportion of the dose. Because the method works on a principle of amounts, the calculation assumes there must be a complete collection of the label excreted in urine. Alternatively, an intermittent dose regimen is used to achieve a steady isotopic state of ^{15}N label entering and exiting the colon, and the *rate* at which label exits the body as urinary urea is calculated. [^{15}N^{15}N]urea is given in smaller multiple doses until isotopic plateau is achieved in urinary urea. When the system is at isotopic equilibrium, the rate of excretion of label into urinary urea can be used to calculate the salvage and retention of label as a proportion of the rate of dose administered. The mathematical model used to calculate urea-N salvage and retention is less complex than for the single dose method, but the study conditions require closer supervision of subjects.

Lactose [^{15}N^{15}N]ureide was given orally as a prime dose followed by *intermittent* doses, in an attempt to quantify the rate at which label exits the colon as ^{15}N-labelled urinary urea during isotopic equilibrium. A prime dose of label was given to shorten the time needed to achieve a isotopic equilibrium. Two forms of ^{15}N-labelled urinary urea were measured. [^{15}N^{15}N]urea represents lactose [^{15}N^{15}N]ureide which has been fermented in the colon, and the urea subsequently absorbed by the body and excreted intact. This allows a calculation of the excretion rate of lactose [^{15}N^{15}N]ureide as urinary [^{15}N^{15}N]urea,

which can be used to calculate [$^{15}N^{15}N$]urea salvaged in the colon and excreted intact. [$^{14}N^{15}N$]urea represents lactose [$^{15}N^{15}N$]ureide which is fermented in the colon, with the urea part subsequently hydrolysed to release ^{15}N-labelled ammonium ions. $^{15}NH_3$ can then be absorbed by the body, reformed to urea in the liver, and excreted in the urine as [$^{14}N^{15}N$]urea. This allows a calculation of the excretion rate of lactose [$^{15}N^{15}N$]ureide as urinary [$^{14}N^{15}N$]urea, which represents the rate at which $^{15}NH_3$ is salvaged in the colon and then excreted as urinary urea.

The flow of label from the colon into the stool must also be considered. It is impractical to try to achieve isotopic equilibrium in the stool compartment, considering the long transit time of label through the colon, and the infrequency of stool collections. Therefore, with respect to stool ^{15}N enrichment, the administration of lactose [$^{15}N^{15}N$]ureide can be viewed as a *single* oral dose, being the sum of the prime and all the intermittent doses. In this way, the total amount of ^{15}N excreted in the stool as a proportion of the total ^{15}N administered was calculated. By measuring the excretion of ^{15}N into the urine and stool, the proportion of salvaged ^{15}N retained in the body nitrogen pool can be calculated.

As a further consideration, it was desirable that the method developed in this chapter could also be applied to calculating the flux of *de novo* synthesised lysine into plasma proteins. In this way, the significance of *de novo* lysine utilisation could be determined in this and in future studies. A preliminary study, detailed in section 2.5.5, demonstrated that isotopic equilibrium in the lysine component of VLDL apoB-100 would be achieved over the current study period. Therefore, it is possible that the method could be used to calculate the flux of *de novo* synthesised lysine into the apoB-100 protein.

4.2 Methods

The protocol followed was the similar to that described in section 3.3.
Nine adult subjects consumed a diet marginally adequate in protein for five days. At 6.00 am on day four of the study, a baseline urine sample was collected

into a container acidified with 20 ml 6 M hydrochloric acid. Subjects were given a oral prime dose of 3.21 mg/kg lactose [$^{15}N^{15}N$]ureide dissolved in deionised water, followed by oral doses of 0.64 mg/kg lactose [$^{15}N^{15}N$]ureide at intervals of 3 h until 9.00 pm. Details of the dosage are described in section 2.5.5. Total urine was collected into acidified containers every three hours for 18 hours following administration of the prime dose. Urinary ammonia and urea concentrations were determined for each urine sample, as discussed in section 2.4.3. About 2 mg urinary urea was isolated from each urine sample by ion-exchange chromatography, and the relative proportions of [$^{15}N^{15}N$] urea and [$^{14}N^{15}N$]urea were determined by isotope-ratio mass-spectrometry (Section 2.4.4). A baseline stool sample was collected from each subject in a polythene bag, and then the total stool passed was collected for up to 72 h following the prime dose. In practise, the collection period varied depending on individual bowel habit and compliance. Stools were analysed for total ^{15}N isotopic abundance by combustion-IRMS. All samples were stored at -20°C before analysis.

4.3 Results

Individual graphs for each subject displaying the profile of urinary [$^{15}N^{15}N$]urea and [$^{14}N^{15}N$]urea isotopic enrichment over the period of study are shown in figures 4.1 to 4.9 (Appendix 4A). The individual plots show the the enrichment in urinary [$^{15}N^{15}N$]urea increased over the first three to six hours, and thereafter maintained a low but significant level of enrichment over the study period. For each individual, the values for [$^{15}N^{15}N$]urea enrichment at 12, 15, and 18 h after the prime dose were taken as representative of a plateau, and used to calculate the proportion of label excreted (Table 4.1). The rate of excretion of lactose [$^{15}N^{15}N$]ureide as urinary [$^{15}N^{15}N$]urea, expressed as a proportion of the rate of ^{15}N administration, was calculated as an average of 5.4 (SD 1.3) % for all the subjects, according to the calculations detailed in section 2.5.6.

Table 4.1 Isotopic enrichment of urinary [$^{15}N^{15}N$]urea at plateau (APE), and the corresponding rate of excretion as a proportion of dose, in nine adult subjects consuming a diet marginally adequate in protein

Subject	*[$^{15}N^{15}N$]urea enrichment*	*% ^{15}N dose excreted*
1	0.02887	5.2
2	0.05995	8.3
3	0.02781	4.8
4	0.03824	6.0
5	0.03360	5.4
6	0.03185	5.0
7	0.02121	3.5
8	0.01981	4.6
9	0.03469	6.1
MEAN (SD)	0.03289 (0.00118)	5.4 (1.3)

It was more difficult to define a plateau level of enrichment in urinary [$^{14}N^{15}N$]urea. For some subjects (1, 3, 4 and 8), a reasonable plateau could be approximated from the last two or three measurements (12, 15 and 18 h after the prime dose). For the remaining subjects (2, 5, 6, 7 and 9), there was no evidence of an isotopic plateau during the study period, and [$^{14}N^{15}N$]enrichment at 18 h was used to represent the closest approximation to a plateau. This value is likely to be a minimum value, and if the study period had been extended it is expected that the true level of enrichment at plateau would have been greater than this. For three subjects who did not achieve a plateau (5,7 and 9), the enrichment of urinary [$^{14}N^{15}N$]urea at 18 h was relatively low, whereas in the other two (2 and 6), enrichment at 18 h was similar to that observed in subjects who achieved isotopic plateau. Individual results are shown in table 4.2. The maximum excretion rate of ^{15}N label as urinary [$^{14}N^{15}N$]urea where plateau was achieved was calculated to be 42 % of the rate of administration lactose [$^{15}N^{15}N$]ureide, according to the calculations detailed in section 2.5.6. On average, 29.5 (SD 8.6) % of the dose was excreted in this form in subjects where plateau could be identified with confidence. Where plateau could not be

identified, an average of 19.9 (SD 9.4) % of the dose was excreted as urinary [^{14}N^{15}N]urea

Table 4.2 Isotopic enrichment of urinary [^{14}N^{15}N]urea (APE), and the corresponding rate of excretion as a proportion of dose, in nine adult subjects consuming a diet marginally adequate in protein

Subject	*[^{14}N^{15}N]urea enrichment*	*% ^{15}N dose excreted*
1	0.12848	23.0
2	0.23032	31.7
3	0.15442	27.0
4	0.16687	25.8
5	0.04278	7.0
6	0.16361	25.6
7	0.11047	18.2
8	0.18157	42.1
9	0.09618	16.8
MEAN (SD)	0.14163 (0.05446)	24.1 (9.9)

Rows where subjects achieved isotopic plateau are shaded

Total ^{15}N excreted in the stool was measured for each subject (Table 4.3). Total collection time of stool was calculated as the time between the prime dose of lactose [$^{15}N^{15}N$]ureide and the last stool collected. The amount of ^{15}N excreted in the stool expressed as a proportion of the total amount of ^{15}N given, was an average of 22.0 (SD 6.9) % for all subjects, according to the calculations detailed in 2.5.6 . The time over which stool was collected varied from 38 to 88 hours. There was no correlation between total ^{15}N excretion in the stool and total time over which stool was excreted. Most subjects tended toward to baseline isotopic enrichment in stool at the end of the collection period.

Table 4.3 Stool collection period, and excretion of ^{15}N in stool as a percentage of the total dose of orally administered lactose [$^{15}N^{15}N$]ureide, in 9 adult subjects consuming a diet marginally adequate in protein.

Subject	*Stool collection (h)*	*% dose excreted*
1	75	23.6
2	77	17.8
3	52	12.7
4	57	17.7
5	88	34.7
6	40	28.8
7	38	25.0
8	78	21.8
9	88	16.0
MEAN (SD)	66 (20)	22.0 (6.9)

Table 4.4 shows the calculated apparent retention of ^{15}N label in the body for the nine subjects studied, taking into account the excretion of ^{15}N in stool. Apparent average retention of ^{15}N label in the body was 48.4 (SD 8.9) % of the total dose of lactose [$^{15}N^{15}N$]ureide administered. However, because the majority of subjects did not achieve urinary [$^{14}N^{15}N$]urea plateau, this value may be an overestimate. Assuming that the rate of excretion of label as [$^{14}N^{15}N$]urea was an average of 30 % in subjects where an isotopic plateau was identified with confidence, retention rate of label was estimated to be greater than 40 % of the dose administered.

Table 4.4 Excretion of ^{15}N into urinary urea and stool, and the calculated percentage of dose retained in the body, after primed/intermittent doses of lactose [$^{15}N^{15}N$]ureide in nine adults consuming a diet marginally adequate in protein

Subject	*Urine ^{15}N (%)**	*Stool ^{15}N (%)+*	*Apparent ^{15}N retained (%)*
1	28.2	23.6	48.2
2	40.0	17.8	42.2
3	31.8	12.7	54.5
4	31.8	17.7	50.5
5	12.4	34.7	52.9
6	30.6	28.8	40.6
7	21.7	25.0	53.3
8	46.7	21.8	31.5
9	22.9	16.0	61.1
MEAN (SD)	29.6 (10.1)	22.0 (6.9)	48.4 (8.9)

* Percentage of total dose of ^{15}N in the form of [$^{15}N^{15}N$] and [$^{14}N^{15}N$]urea

+ Total ^{15}N excreted in the collected stool for each individual

Rows where subjects achieved isotopic plateau in both [$^{14}N^{15}N$] and [$^{15}N^{15}N$] urea are shaded

4.4 Discussion

The aim of this study was to use lactose [$^{15}N^{15}N$]ureide as a *non-invasive* means to *quantify* the fate of urea-N salvaged in the colon of nine adult subjects consuming a diet marginally adequate in protein. Three measurements were made.

a The rate of excretion of lactose [$^{15}N^{15}N$]ureide as [$^{15}N^{15}N$]urea in the urine

b The rate of excretion of lactose [$^{15}N^{15}N$]ureide as [$^{14}N^{15}N$]urea in the urine

c The total amount of lactose [$^{15}N^{15}N$]ureide excreted as ^{15}N in the stool

An increase in urinary [$^{15}N^{15}N$]urea enrichment was apparent in all subjects after 3 hours following the administration of the prime dose. As stated in chapter 3, transit time for material from the oral cavity to the entrance of the colon has been reported in the range of 4.3 to 9 hours. Heine *et al* (1995) reported that isotopically labelled compound appeared in the urine 1 to 2 hours after a single oral dose of lactose [^{13}C]ureide in one adult, and totalled 9 % of the dose. This was identified to be in the form of glucose [^{13}C]ureide, which was presumed to be lactose [^{13}C]ureide that had undergone partial enzymatic cleavage via β-galactosidase (*EC 3.2.1.23*) in the upper gut before being absorbed and excreted in urine. It is possible that urinary glucose [$^{15}N^{15}N$]ureide was making a contribution to the early rise in urinary [$^{15}N^{15}N$]urea enrichment observed in the present study. [$^{15}N^{15}N$] gas is released from [$^{15}N^{15}N$]urea after reaction with lithium hypobromite during sample preparation for mass spectrometry. If this reaction also caused labelled nitrogen gas to be liberated from glucose [$^{15}N^{15}N$]ureide, this would contribute to apparent [$^{15}N^{15}N$]urea enrichment.

Another possibility is that transit time from mouth to ileocoecal valve is quicker in the present subjects than has been reported in other studies, resulting in earlier bacterial hydrolysis of lactose [$^{15}N^{15}N$]ureide, and release of [$^{15}N^{15}N$]urea. This would correlate with the initial rise in breath $^{13}CO_2$ enrichment in all subjects 3 h after a single dose of lactose [^{13}C]ureide (section 3.4). Similarly, Wutzke and

colleagues (1997) have recently reported an average ileocoecal transit time of 3.1 (SD 1.4) h in 12 healthy adults after a single oral dose of lactose [^{13}C, ^{15}N]ureide, with reported transit time as low as 1.5 h in 3 of the subjects.

Urinary [$^{15}N^{15}N$] reached isotopic plateau in all subjects by 6 hours, as determined by visual inspection of the data, and the calculated rate of excretion at equilibrium was an average of 5.4 % of the rate of administration of dose. This is similar to the total excretion of a single dose of [$^{15}N^{15}N$]urea instilled into the left and right functioning colon through a colonoscope as urinary [$^{15}N^{15}N$]urea, being an average of 4.1 % and 6.2 % respectively (Moran and Jackson, 1990*b*). The present study provides further evidence that the colon is permeable to intact urea, and also that only a small proportion of label is excreted in this form, the rest presumably undergoing further metabolism through the actions of the colonic microflora

An isotopic plateau in urinary [$^{14}N^{15}N$]urea enrichment was identified in four subjects (1,3,4 and 8) over the period of study. These subjects were also quicker to reach a measured peak of breath $^{13}CO_2$ excretion following administration of a single oral dose of lactose [^{13}C]ureide (average of 10.8 h) than subjects who did not attain plateau (average of 15.8 h), as described in section 3.4. It is presumed that the differences in the time taken to achieve plateau enrichment between the subjects are a reflection of differences in gastrointestinal transit time, assuming the overall rates of fermentation, hydrolysis and excretion of lactose-ureide were similar. Lengthening the study protocol by another six hours would have probably allowed all subjects to come into isotopic equilibrium in urinary [$^{14}N^{15}N$]urea, and this should be taken into account in future studies. The rate of excretion of rate of lactose [$^{15}N^{15}N$]ureide as [$^{14}N^{15}N$]urea was calculated to be an average of about 30 % of the dose in subjects where plateau was achieved.

In considering the amount of label excreted into the stool, it is also important to consider the *form* in which label enters and exits the colon. Mean total ^{15}N excretion in stool (22.0 %) was higher than previously reported by Moran and Jackson (1990*b*) in 12 adult subjects who received a single dose of

[^{15}N^{15}N]urea instilled directly into the colon (>4 % after 72 h). However, in the present study, ^{15}N label was presented to the colon as lactose [^{15}N^{15}N]ureide. When lactose [^{15}N^{15}N]ureide enters the colon, the chemical bond between lactose and the urea moiety must be cleaved before the [^{15}N^{15}N]urea can be hydrolysed. The hydrolysis of lactose-ureide is thought to be catalysed by two enzymes, allantoate-amidohydrolase (*EC 3.5.3.9*) and allantoate-amidinohydrolase (*EC 3.5.3.4*), whereas urea hydrolysis is catalysed by urease (*EC 3.5.1.5*; Wutzke *et al*, 1997). Therefore, the rate of hydrolysis of the urea part of lactose-ureide is dependant on the rate of hydrolysis of lactose-ureide. Assuming that about 4 % of a single dose of [^{15}N^{15}N]urea presented directly to a normal colon is excreted as ^{15}N in the stool, as reported by Moran and Jackson (1990*a*), it can be further assumed that the difference in total ^{15}N excretion in stool between the studies (about 18 % of the total dose administered on average) represents lactose [^{15}N^{15}N]ureide which has not undergone hydrolysis in the colon, and has passed through the gut intact. Additionally, the data in chapter 3 demonstrate that on average, 79.4 % of a single dose of lactose [^{13}C]ureide was excreted on breath of the same subjects after 48 h. This then allows a distinction to be made between the total *administered* dose of lactose [^{15}N^{15}N]ureide, and the total dose acted upon by the colonic bacteria and subsequently *available* to the body. Further analysis of the stool fractions obtained in the present study shows that, on average, about 85 % of the label (about 19 % of total dose) is associated with the non-bacterial fraction, and 15 % (about 3 % of total dose) with the bacterial fraction (A. Hounslow, personal communication). Wrong and colleagues (1970) have reported that detectable concentrations of urea were not found in the faecal dialysate of normal subjects, with only a small amount (11.2 mmole/l) excreted as ammonia. Therefore, it can be estimated that most of the 85 % of the stool label associated with the non-bacterial fraction was excreted as lactose [^{15}N^{15}N]ureide, and this is similar to that which can be estimated by comparing the present results to those of Moran and Jackson (1990*b*). The remaining 15 % of stool ^{15}N is assumed to be in the form of products of bacterial metabolism, such as amino acids and nucleic acids.

Assuming then that 81 % of the administered label (total dose - 19 % assumed as faecal lactose-ureide) was available to the body, the present data can subsequently be recalculated for subjects who achieved isotopic plateau in both urinary [$^{15}N^{15}N$] and [$^{14}N^{15}N$]urea (Table 4.5)

Table 4.5 Comparison between the urinary and faecal excretion of lactose [$^{15}N^{15}N$]ureide in subjects 1,3,4 and 8, expressed as a proportion of the total *administered* dose and the dose *available* to the body

	% dose administered	*% dose metabolized*
Urinary [$^{15}N^{15}N$]urea	5.2	6.4
Urinary [$^{14}N^{15}N$]urea	29.5	36.4
Stool ^{15}N (Bacterial fraction)	2.8	3.3
TOTAL EXCRETION OF AVAILABLE DOSE		46.1

It is concluded that, on average, the rate of ^{15}N retention in the body was *about half* of the rate of lactose [$^{15}N^{15}N$]ureide administered which was subsequently available to the body. This value is lower than that reported by Moran and Jackson, who observed a retention of nitrogen within the body pool of 74 ± 16 % in the right colon and 82 ± 14 % in the left colon of normal adult male patients after 72 h following a single dose of [$^{15}N^{15}N$]urea instilled directly into the colon, and may be as a result of different diets, and the metabolic state of these subjects, compared with the present study.

In summary, this chapter has demonstrated that lactose [$^{15}N^{15}N$]ureide provides a useful way to non-invasively quantify the fate of salvage of urea-N in normal individuals. Further, the method which has been developed has potential to help determine the extent to which *de novo* lysine synthesised by the colonic bacteria is utilised by the body.

Appendix 4A Figures

Figure 4.1 Enrichment of urinary [$^{15}N^{15}N$] and [$^{14}N^{15}N$]urea following a prime and intermittent oral doses of lactose [$^{15}N^{15}N$]ureide in subject 1

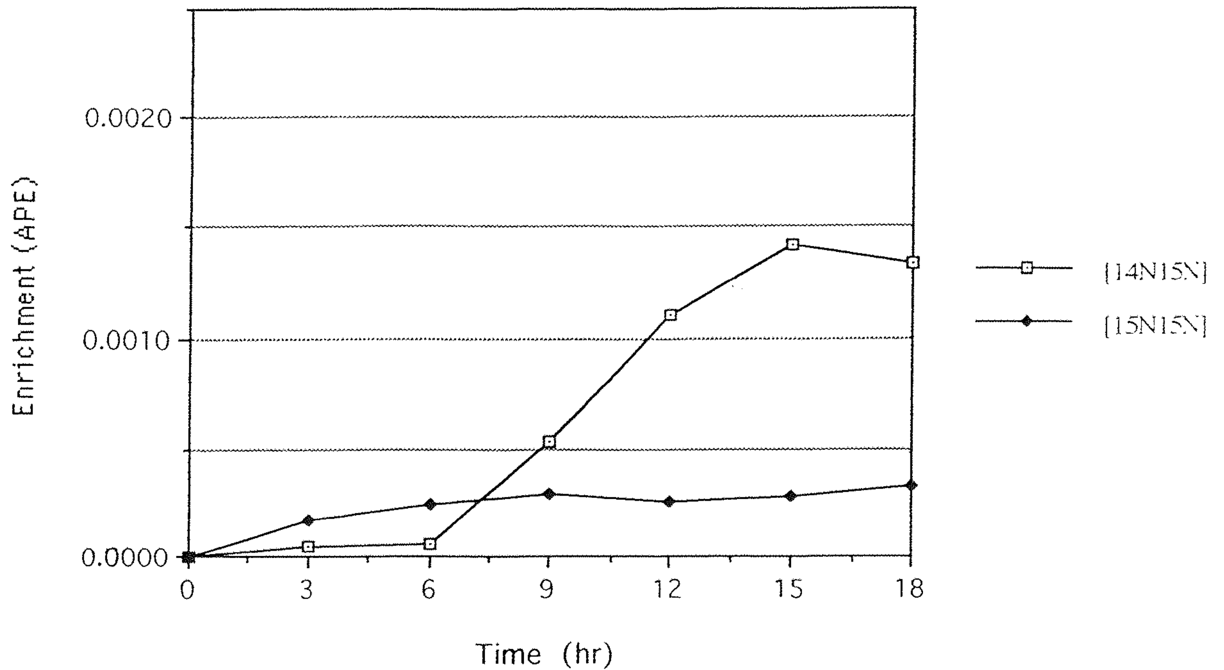


Figure 4.2 Enrichment of urinary [$^{15}N^{15}N$] and [$^{14}N^{15}N$]urea following a prime and intermittent oral doses of lactose [$^{15}N^{15}N$]ureide in subject 2

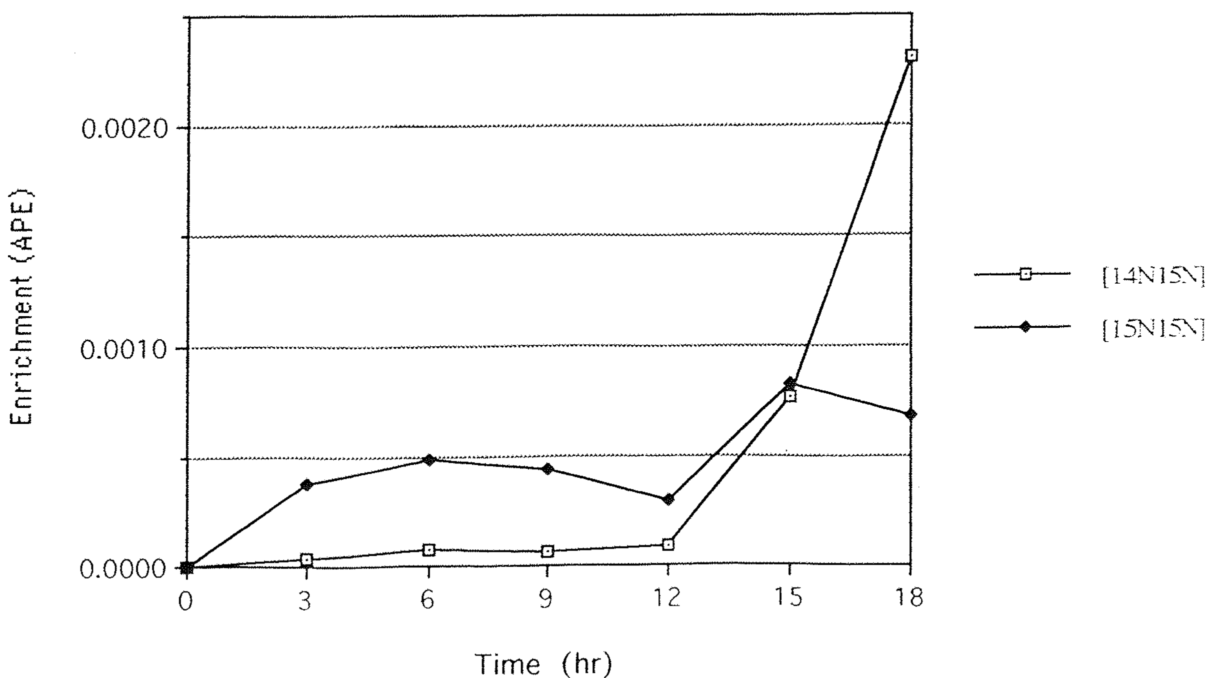


Figure 4.3 Enrichment of urinary [$^{15}N^{15}N$] and [$^{14}N^{15}N$]urea following a prime and intermittent oral doses of lactose [$^{15}N^{15}N$]ureide in subject 3

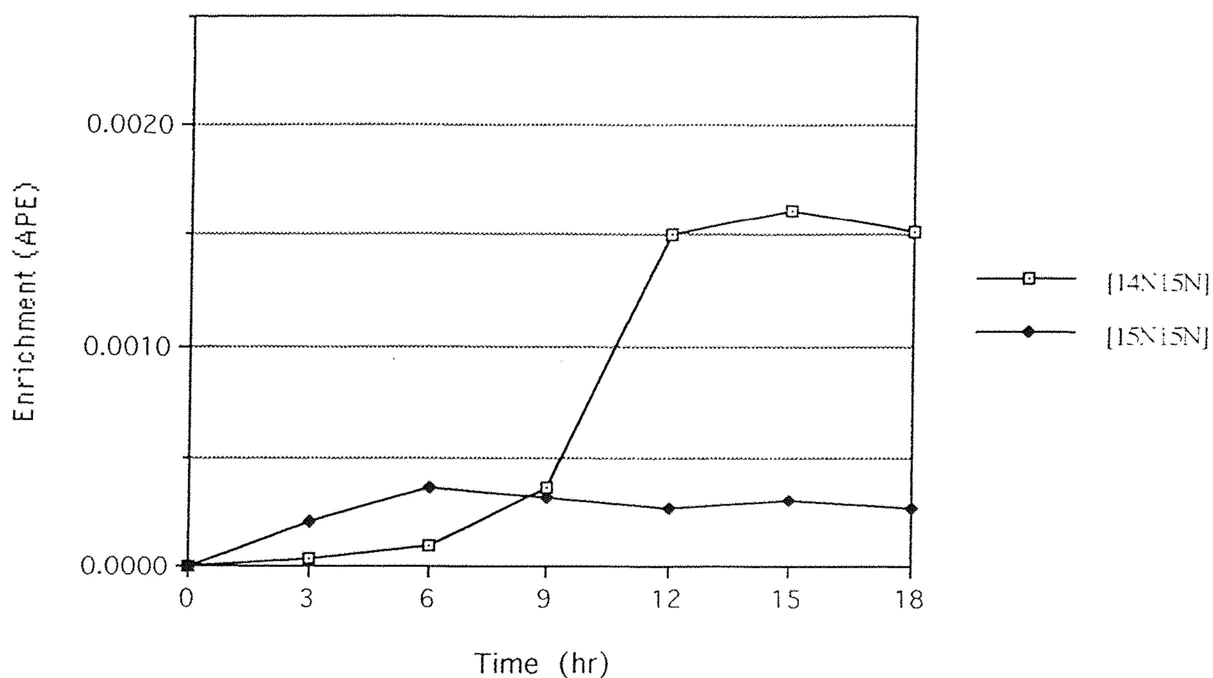


Figure 4.4 Enrichment of urinary [$^{15}N^{15}N$] and [$^{14}N^{15}N$]urea following a prime and intermittent oral doses of lactose [$^{15}N^{15}N$]ureide in subject 4

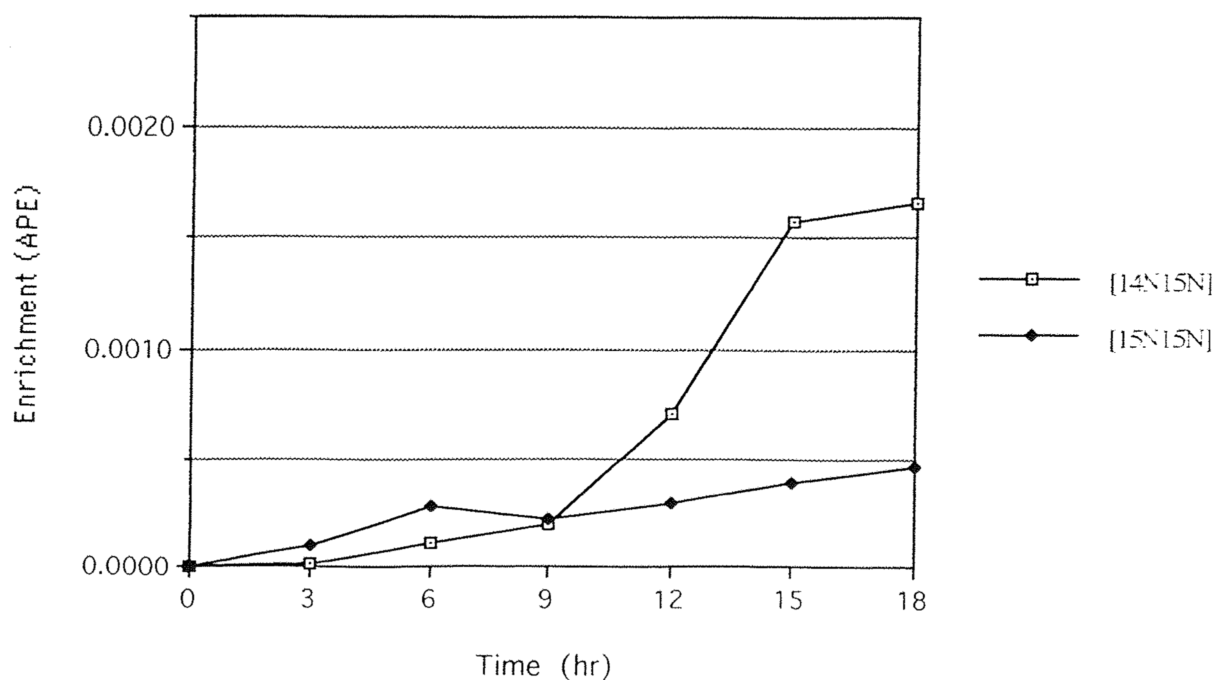


Figure 4.5 Enrichment of urinary [$^{15}N^{15}N$] and [$^{14}N^{15}N$]urea following a prime and intermittent oral doses of lactose [$^{15}N^{15}N$]ureide in subject 5

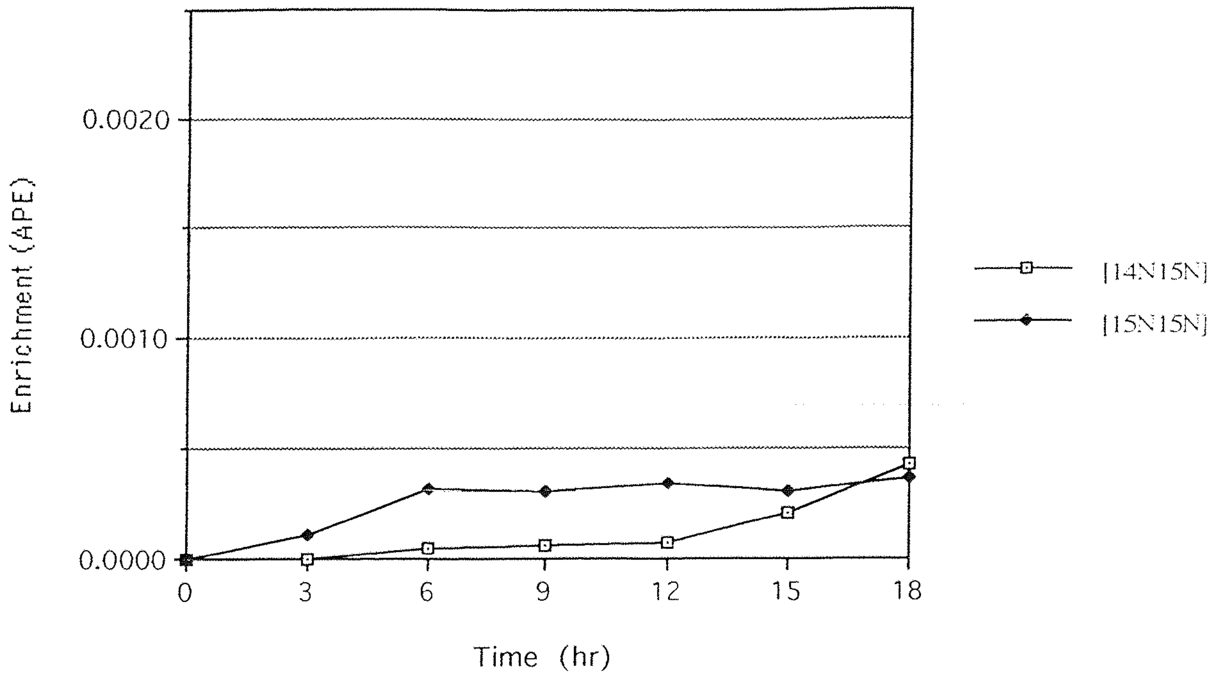


Figure 4.6 Enrichment of urinary [$^{15}N^{15}N$] and [$^{14}N^{15}N$]urea following a prime and intermittent oral doses of lactose [$^{15}N^{15}N$]ureide in subject 6

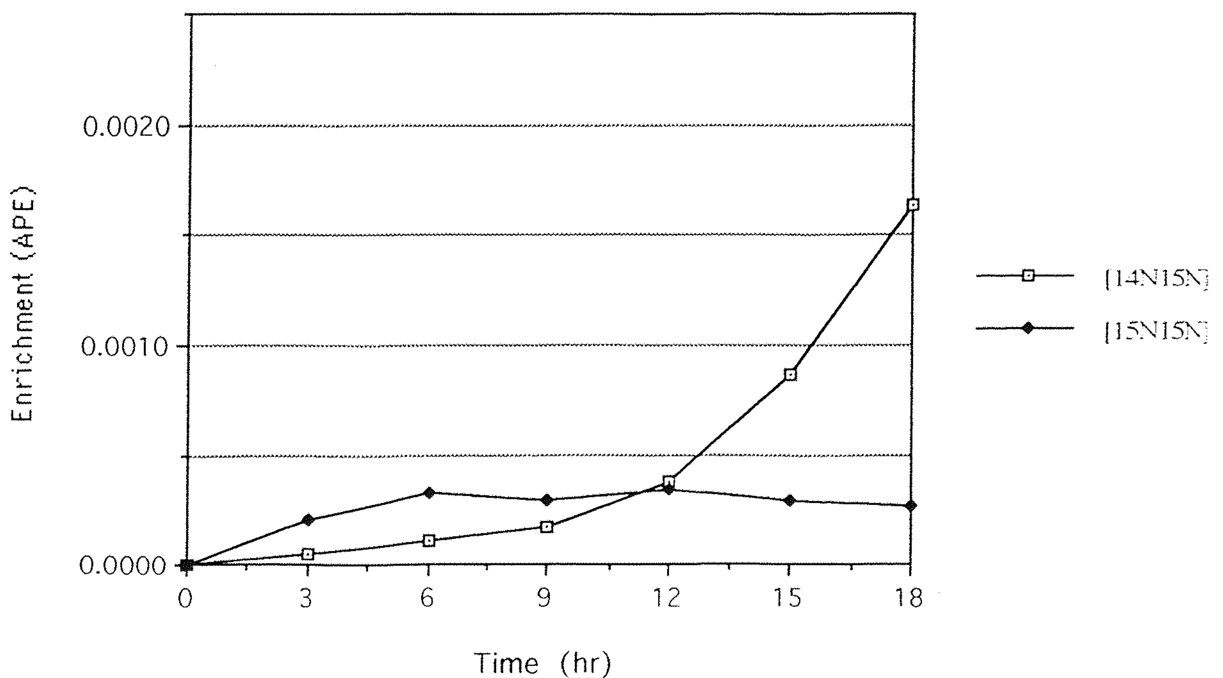


Figure 4.7 Enrichment of urinary [$^{15}N^{15}N$] and [$^{14}N^{15}N$]urea following a prime and intermittent oral doses of lactose [$^{15}N^{15}N$]ureide in subject 7

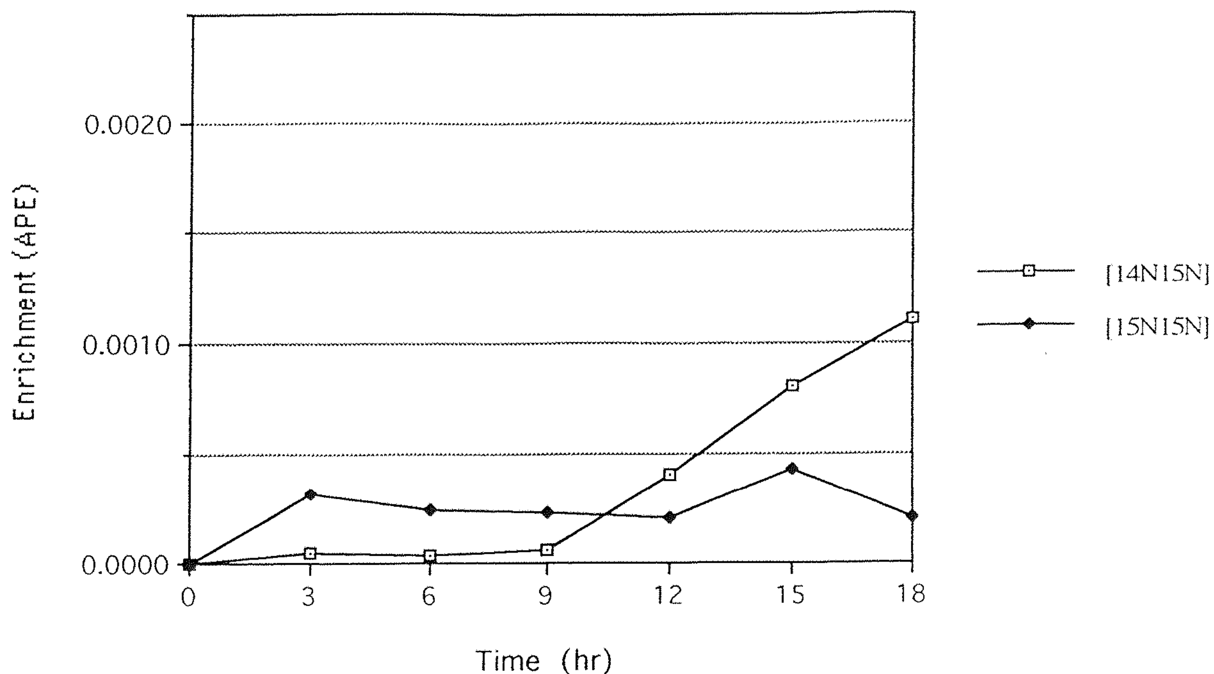


Figure 4.8 Enrichment of urinary [$^{15}N^{15}N$] and [$^{14}N^{15}N$]urea following a prime and intermittent oral doses of lactose [$^{15}N^{15}N$]ureide in subject 8

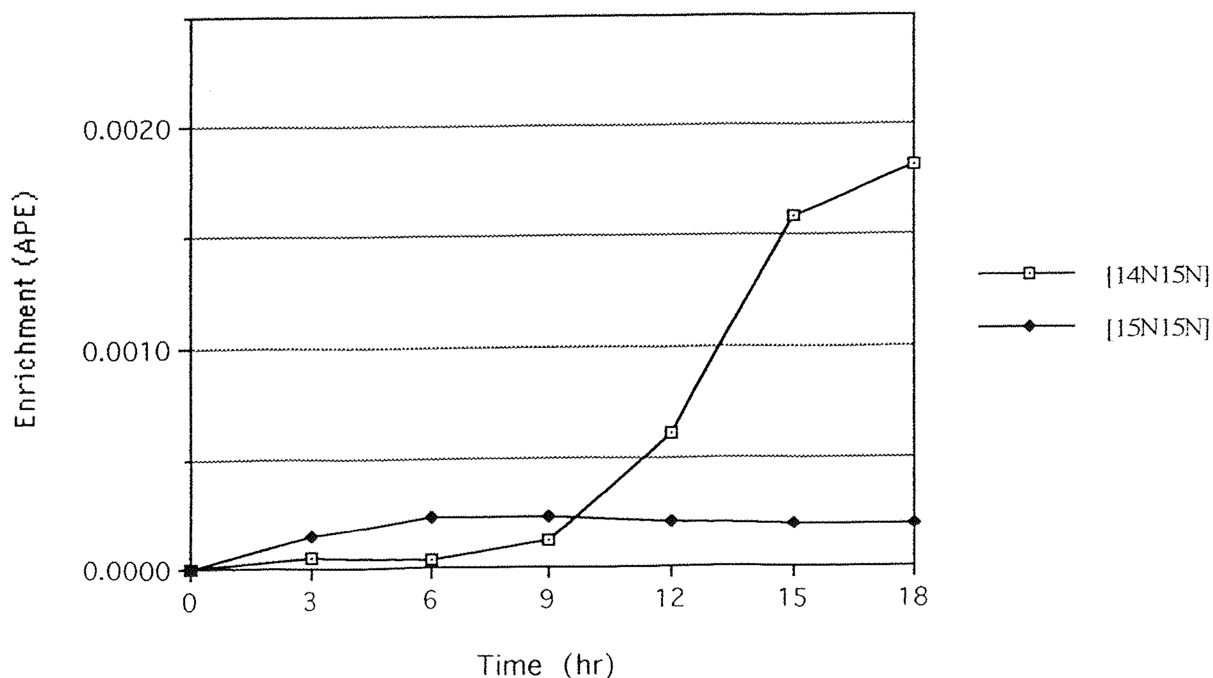
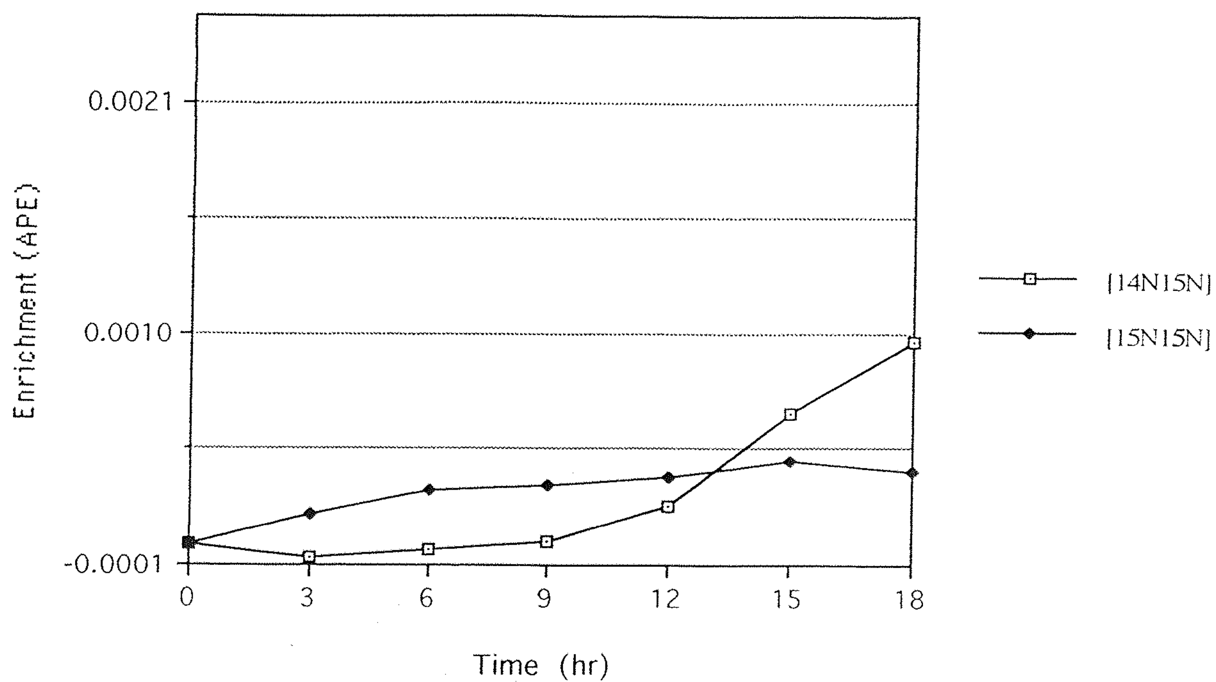


Figure 4.9 Enrichment of urinary [$^{15}N^{15}N$] and [$^{14}N^{15}N$]urea following a prime and intermittent oral doses of lactose [$^{15}N^{15}N$]ureide in subject 9



CHAPTER 5

Isolation of lysine from apoB-100 hydrolysates and measurement of ^{15}N isotopic enrichment

5.1 Introduction

The aim of this chapter was to develop a method to isolate lysine from the plasma protein apolipoprotein B-100 in a quantity sufficient for analysis of ^{15}N isotopic enrichment with mass spectrometry.

The form in which urea-N is salvaged in the colon and retained in the body is still unclear. The suggestion that essential amino acids formed *de novo* by the colonic bacteria may be available to the body is of great potential significance. Evidence to support this suggestion has existed since 1968, when Giordano and colleagues demonstrated significant ^{15}N enrichment in the lysine fraction of albumin in patients with chronic renal failure consuming a low protein diet, who were given oral doses of [^{15}N]urea. The potential for this to occur in normal adults when the dietary protein supply is limiting or when the metabolic demand for nitrogen increases, is essential to explore if a clearer understanding of adult nitrogen requirements is to be obtained, and has a important bearing on the current controversy surrounding adult essential amino acid requirements.

Lysine is one of only two essential amino acids whose carbon skeletons can neither be synthesised nor aminated in the body. During studies using stable isotopes, transfer of ^{15}N from non-protein sources, such as [^{15}N]ammonium or [$^{15}N^{15}N$]urea, to lysine is only possible through the metabolic activity of the bacteria in the colon. Detection of lysine significantly enriched with ^{15}N after administration of ^{15}N-labelled non-protein compounds therefore indicates *de novo* synthesis of lysine in the colon. Furthermore, detection of [^{15}N]lysine enrichment in body protein indicates utilisation of *de novo* synthesised lysine. Despite accumulating evidence to suggest that *de novo* synthesised lysine is utilised by the body under conditions when dietary protein supply is limited and/or demand is increased (Tanaka *et al*, 1980; Yeboah *et al*, 1996), methodological limitations have not allowed a direct quantification of the phenomenon.

Chapter 4 demonstrated that lactose [$^{15}N^{15}N$]ureide is a suitable vehicle for delivering a known dose of [$^{15}N^{15}N$]urea to the colon intact, and that the flux of ^{15}N into urine can be quantified in a steady isotopic state allowing a calculation of the label retained in the body nitrogen pool. A further step was to investigate whether a proportion of salvaged urea-N is retained in the body as *de novo* synthesised lysine. In calculating the significance of *de novo* lysine utilisation using a stable isotope tracer methodology, measurements of lysine enrichment should ideally be taken from samples as near as possible to the site of protein synthesis. Plasma proteins are synthesised primarily in hepatic cells and then secreted into plasma. They are formed from amino acids supplied from the hepatic amino acid pool, which in turn is supplied with amino acids from the diet, protein degradation, and *de novo* synthesis. Direct measurement of the hepatic amino acid pool can only be achieved by taking samples of liver, a technique which has limited application. Recently, Reeds and colleagues (1992) have developed an alternative method utilising apolipoprotein B-100 (apoB-100) from the VLDL compartment of plasma. This method is based upon two main principles. First, over 98% of apoB-100 is of hepatic origin, and so the pattern of isotopic labelling in apoB-100 during an isotopic study allows a minimally invasive measure of the pattern of labelling in the hepatic amino acid pool from which apoB-100, and perhaps other plasma proteins, are derived. Second, apoB-100 has a relatively short-half life in the intravascular space and so reaches isotopic equilibrium

during isotopic infusion within a period compatible with conventional protocols (Reeds *et al*, 1992). A pilot study described in section 2.5.5 demonstrated that the lysine fraction of apoB-100 attained an isotopic plateau within 4 to 6 hours following oral doses of [^{15}N]lysine, similar to what has been reported using other stable isotopes (Venkatesan *et al*, 1990; Reeds *et al*, 1992; Motil *et al*, 1994), and so is compatible with the lactose [$^{15}N^{15}N$]ureide protocol. During the study detailed in chapter 4, two blood samples were taken from each subject, at 6.00 am on day 4 before administration of lactose [$^{15}N^{15}N$]ureide and at 18 h following the prime dose, for the purpose of attempting to quantify the flow of *de novo* lysine into plasma proteins under these study conditions.

In order to quantify the flow of ^{15}N into apoB-100 lysine, it is necessary to separate lysine from the protein in a quantity which can be further analysed accurately for isotopic enrichment. The first challenge relates to separation of lysine from a protein hydrolysate. Amino acid separation was developed by Moore and colleagues in the 1950's, and the basic principle of the method is still applied in modern automated amino acid analysers. Amino acids are separated on a cation exchange resin column and eluted with a gradient of acidic citrate buffers which are applied stepwise. The column effluent is derivatised by mixing either with ninhydrin for colorimetric detection of amino acids, or with *o*-phthalaldehyde (OPA) for fluorescent detection (Walker and Mills, 1995). More recently, new techniques, including high performance liquid chromatography (HPLC), have been developed to analyse amino acid mixtures with the main objectives of *increasing detection sensitivity* and *decreasing analysis time* (Lavi and Holcenberg, 1986). A number of these HPLC methods involve derivatization of amino acids before they are separated in order to increase the precision and sensitivity of the method (Walker and Mills, 1995). The eluted derivatives can be detected by absorption of light at a specific wavelength, or by using a fluorimeter. Among the derivatization reagents, phenylisothiocyanate (PITC) may be used to produce PTC(phenylthiocarbamyl)-amino acids, stable derivatives that can be separated with good resolution. Separation of amino acids by HPLC is typically used as an analytical procedure to quantify amino acids in small samples of biological fluid (1-10 pmol), and derivatization has largely become an automated process. For the purposes of the thesis, we wished to

isolate lysine in a quantity which could then be analysed for isotopic enrichment by mass-spectrometry. This meant that the whole derivatization and separation process had to be scaled up. Pre-derivatization of amino acids with PITC followed by separation PTC-lysine using a reverse-phase semi-preparative column appeared to offer a suitable method which could be scaled up relatively easily.

The second challenge concerns analysis of lysine samples for changes in ^{15}N isotopic enrichment. There are a number of widely established mass spectrometry techniques that can be used to detect changes in isotopic enrichment. Gas-chromatography mass-spectrometry (GCMS) is able to accurately measure changes in enrichment in the order of 0.05 APE in small samples (≤1 nmol), whereas isotope-ratio mass-spectrometry (IRMS) can accurately detect much smaller changes in enrichment, as low as 0.0002 APE, but requires a larger sample size (>1 umol; Preston and Slater, 1994). A relatively new technology, combustion-IRMS, can theoretically measure small change in isotopic enrichment (as low as 0.0008 APE; Brookes, 1990) in relatively small samples of nitrogen. Since we had both a limited supply of biological material in the form of apoB-100 (typically 7 to 8 mg/100 ml plasma, equivalent to about 2.5 uM) together with a small predicted change in ^{15}N isotopic enrichment due to the dilution of our label in a large colonic and body nitrogen pool, c-IRMS seemed to be a suitable method to measure changes in ^{15}N enrichment in lysine isolated from apoB-100.

Detailed in this chapter are studies that were undertaken to:

a Develop a method to manually derivatize a mixture of 20 amino acids with PITC, and subsequently separate and collect the PTC-lysine fraction using HPLC

b Investigate the efficiency of the derivatization and separation procedures developed in part *a*

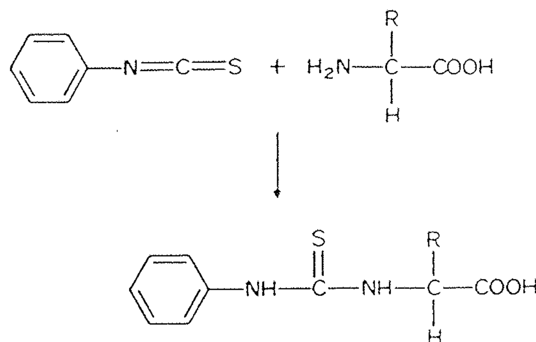
c Determine the suitability of c-IRMS to measure changes in ^{15}N isotopic enrichment in samples of apoB-100 lysine

d Separate lysine from samples of VLDL apoB-100 collected during a clinical trial, and analyse changes in ^{15}N isotopic enrichment with c-IRMS

5.2 Methods

Descriptions of the derivatization, separation, and preparation methods employed in this chapter are presented first. Separation of lysine pre-derivatized with PITC from a mixture of amino acid using HPLC was explored. PITC causes a large non-polar chemical group to attach to the N-terminus of an amino acid under alkaline conditions (Figure 5.1).

Figure 5.1 The formation of phenylthiocarbomyl derivatives of amino acids (after Li and Kim, 1993)



The reaction is complete after 20 min at room temperature (Bidlingmeyer *et al*, 1984), and the resultant PTC-amino acid derivatives are stable for up to two months at -20°C (Walker and Mills, 1995). The derivatives can be separated using HPLC, and detected by absorption of light at 254 nm. This technique was first demonstrated by Koop *et al* (1982), and subsequently modified for the separation of free amino acids from acid-hydrolysed proteins (Bidlingmeyer *et al*, 1984; Heinrikson and Meredith, 1984). It has been shown to be a rapid, efficient, sensitive and specific technique for the analysis of primary and secondary amino acids. The derivatization and separation methods have been validated, and found to be a suitable alternative to traditional ion-exchange chromatography for analysing amino acids in physiological fluids, as well as offering the advantages

of speed of analysis, sensitivity, and versatility of equipment over conventional methods (Davey and Ersser, 1990).

5.2.1 Derivatization of amino acids

The derivatization method employed was a modification of that used by Bidlingmeyer and colleagues (1984). Reagents used in derivatization are detailed in appendix 5A. 100 ul of each sample to be derivatized were pipetted into glass screw-top vials and dried under vacuum. 200 ul of coupling reagent were added to each vial, mixed by vortex, and dried. 200 ul of derivatization reagent were added to each vial and mixed. The vials were sealed, left for 20 min at room temperature, and then dried under vacuum and stored at -20°C before further analysis. Oxygen interferes with the derivatization process, and particular care was taken to avoid samples coming in contact with air. The derivatization reagent was prepared daily under a continuous stream of nitrogen gas and each sample vial was purged with nitrogen and sealed after the reagent had been added. PITC was purchased in 1 ml ampoules sealed under argon and stored at 4°C, and a fresh ampoule was used in each set of reactions.

5.2.2 Separation of amino acids by HPLC

PTC-amino acid derivatives were separated on a reverse-phase HPLC column running a graded bi-solvent system. Conditions for the separation of samples were similar the those employed by Bidlingmeyer *et al* (1984). Details of equipment and solvent gradients can be found in appendix 5A. A semi-preparative column was used (10 X 250 mm) in the current study, as opposed to an analytical column, which allows for the separation of up to 2 mg amino acids each run. Samples were dissolved in a carrier solvent and loaded onto the column with a glass syringe through a Rheodyne valve connected to a 20 ul injection loop. Eluent was analysed for absorption at 254 nm with a UV detector, and information was relayed to a personal computer (PC) and displayed on-screen. Data were stored on magnetic disk and hard-copies were produced on a dot-matrix printer. The column was calibrated every day with 20 ul of a PTC-lysine standard to identify the elution time. Eluent was collected into glass screw-top

vials, dried under vacuum, and stored at -20°C until further analysis. The solvent system was found to be adequate to resolve lysine from a mixture of 20 amino acids and the run time was sufficient to prevent any cross-contamination between runs.

5.2.3 Preparation of lysine samples for mass spectrometry

Samples of PTC-lysine separated by HPLC were prepared for analysis of ^{15}N enrichment by c-IRMS. 20 ul carrier solvent were added to each dried PTC-lysine sample, mixed by vortex, and transferred to smooth-walled tin capsules (Elemental Microanalysis Ltd., Devon, UK) which had been placed into eppendorf tubes for support. Samples were dried under vacuum, and the capsules were carefully removed from the eppendorf tubes and folded using tweezers. Samples were introduced into a Automated Nitrogen and Carbon Analysis (ANCA) system linked to a 20/20 mass-spectrometer (Europa Scientific, Crewe, UK) and ^{15}N isotopic enrichment in each sample was determined. Data were relayed to a PC, stored onto magnetic disk, and a hard-copy was produced on a dot matrix printer.

5.3 Separation and identification of PTC-lysine

This section describes the protocol employed to separate and identify PTC-lysine from a mixture of PTC-amino acid mixtures using HPLC. Three amino acids solutions were made up at concentrations of 50 mmol from purified amino acid standards:

a a solution containing all 20 amino acids (20AA mix)

b a solution containing 19 amino acids without lysine (19AA mix)

c a solution of lysine only

20 ul aliquots of each amino acid solution were derivatized with PITC in triplicate as detailed in section 5.2.1. 100 ul of carrier solvent were added to each sample

vial and mixed by vortex. 20 ul samples were separated by HPLC described in section 5.2.2.

Figures 5.2 to 5.4 show the elution profiles for the 20AA mix, 19AA mix and lysine samples for a typical run. Time in minutes is shown on the *x* axis and an arbitrary peak height value on the *y* axis. Peaks with a mean retention time of 36.1 (SD 0.1) and 36.0 (SD 0.0) min were resolved in the 20AA mix and lysine samples respectively, but were absent from the 19AA mix. 14 other major peaks were identified in both the 20AA and 19AA mixture at similar time points.

Figure 5.2 Typical elution profile of a mixture of 20 PTC-amino acids separated on a reverse-phase HPLC column using a graded bi-solvent system

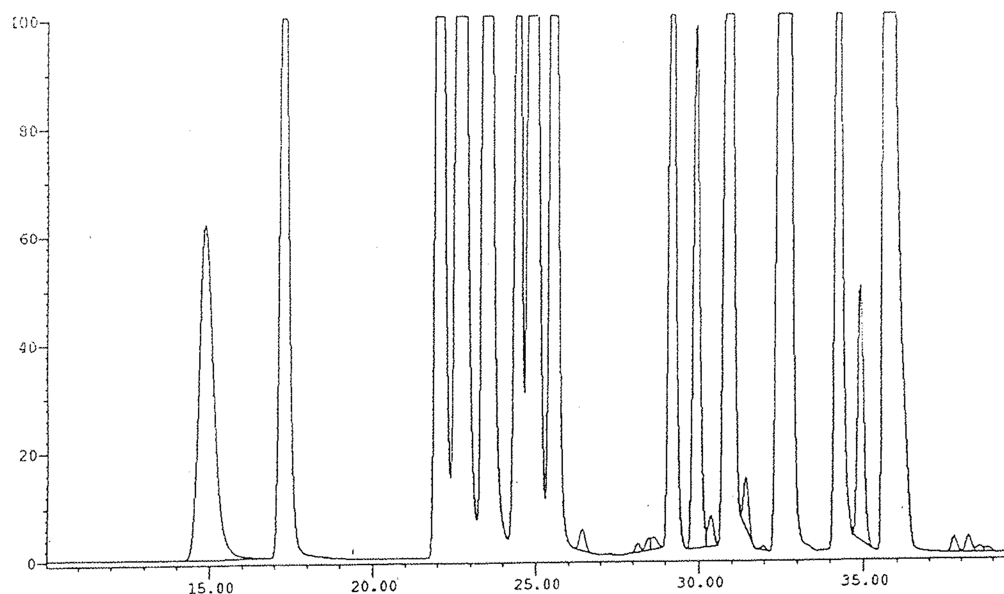


Figure 5.3 Typical elution profile of a mixture of 19 PTC-amino acids (no lysine) separated on a reverse-phase HPLC column using a graded bi-solvent system

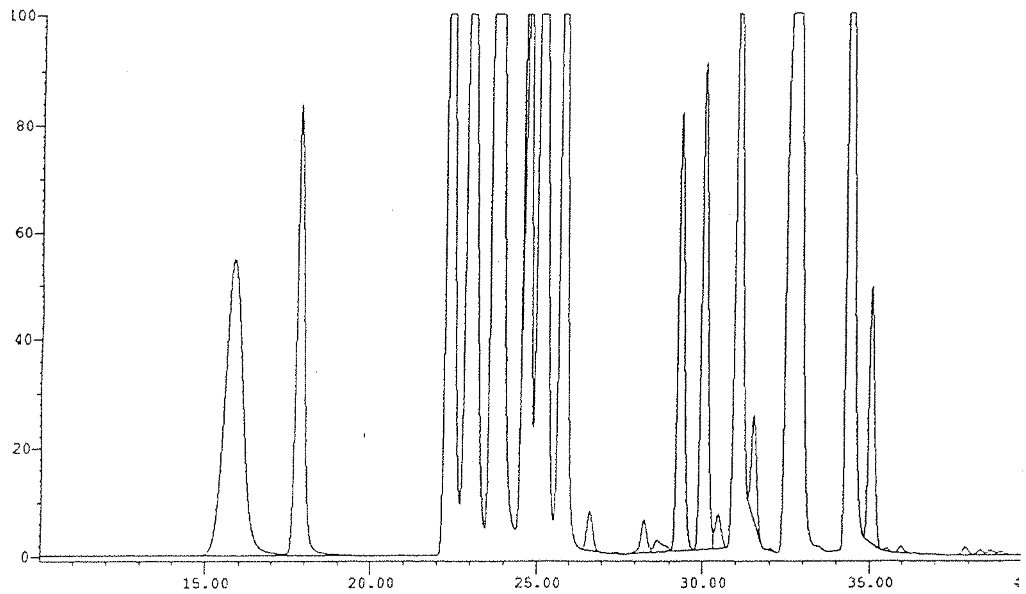
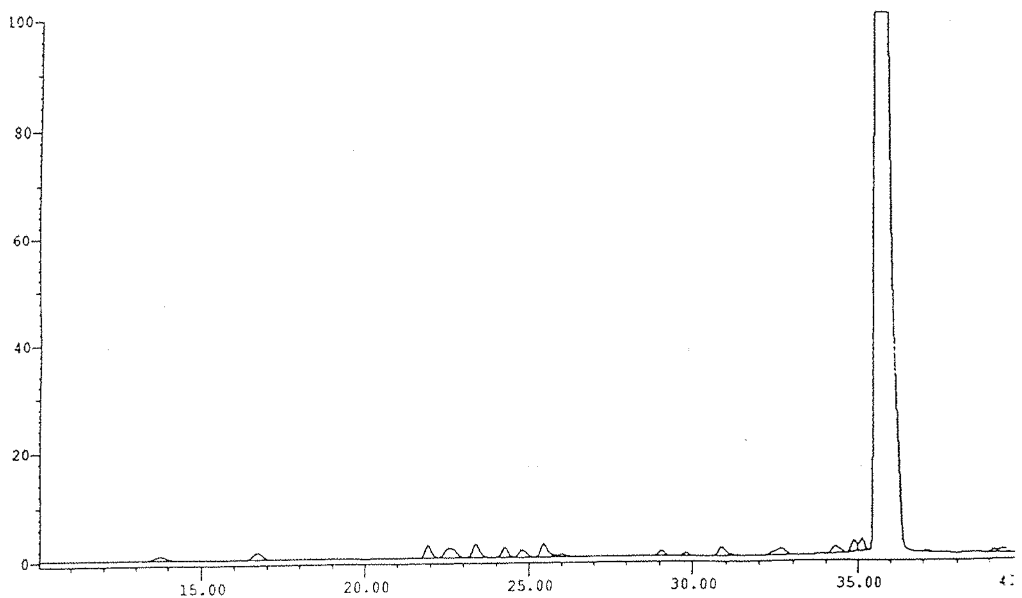


Figure 5.4 Typical elution profile of PTC-lysine separated on a reverse-phase HPLC column using a graded bi-solvent system



It was concluded that the peaks resolved at 36.1 min in the 20AA mix and 36.0 min in the lysine solution corresponded to the PTC-lysine derivative. The PTC-lysine peak was well resolved and had a consistent elution time, making the method suitable for collection of the lysine derivative while avoiding contamination from other amino acids.

5.4 Measured efficiency of the derivatization and separation procedures using [^{3}H]lysine

It was necessary to determine the overall efficiency of amino acid derivatization and separation by observing any loss of lysine sample during these processes. The efficiency of the system has an important bearing on the amount of lysine which is required in order that the derivatized and separated sample collected is of a sufficient quantity to accurately measure changes in isotopic enrichment using c-IRMS. A radioactive labelled stock solution was prepared, containing 20 amino acids at a concentration of 50 mmol plus 0.1 % [^{3}H]lysine solution (3.7 x 10^7 Bq/ml; Cambridge Isotope Laboratories, Cambridge, MA).
20 ul aliquots of stock were added to 10 ml scintillation fluid in triplicate and radioactivity was measured using a scintillation counter. 200 ul stock were derivatised with PITC, and 20 ul aliquots were measured for radioactivity in triplicate. 20 ul aliquots of derivatised stock were also introduced onto the HPLC column in triplicate, and the eluted peak corresponding to PTC-lysine was collected from each sample and measured for radioactivity.

Radioactivity in the separated samples were detected in peaks with a mean elution time of 35.7 (SD 0.3) min, corresponding to the previously determined elution time of PTC-lysine. Table 5.1 shows the radioactivity for samples at each stage of the method, and values expressed as a proportion of the stock solution value. The results show that, on average, about 25% [^{3}H]lysine was lost during the derivatization procedure, and a further 14% through separation by HPLC and collection. Variation between samples was greater after derivatization and separation than in the stock samples. The results suggest that the overall

efficiency of derivatization and separation of lysine using HPLC is about 60 % as determined using [^{3}H]lysine.

Table 5.1 Mean radioactivity of stock, derivatized and separated samples of a radioactive stock 20AA mix, expressed as absolute values and as a proportion of the stock values.

	Radioactivity (d.p.m.)		*Percentage of initial sample*	
	Mean	*SD*	*Mean*	*SD*
Stock	33645	888	100	3
Derivatized	25313	6618	75	26
Separated	20481	3706	61	18

5.5 Accuracy of measurement of ^{15}N enrichment in PTC-lysine with regard to sample size and isotopic enrichment

A further challenge was to investigate whether c-IRMS could accurately measure small changes in ^{15}N isotopic enrichment in PTC-lysine, in an amount suitable for the purposes of the thesis. During the clinical trial described in chapter 4, two 20 ml blood samples were taken from each patient, at time 0 and 18 h after a prime and intermittent oral doses of lactose [$^{15}N^{15}N$]ureide, for the purpose of quantifying the flow of *de novo* lysine into VLDL apoB-100. The typical concentration of apoB-100 in plasma is 0.07 to 0.08 g/l on average. About 10 ml plasma were isolated from each blood sample taken from subjects, so theoretically there was about 700 to 800 ug apoB-100 in each sample. Assuming an equal distribution of 20 amino acids in apoB-100, this is equivalent to about 35 to 40 ug lysine, which is in turn equivalent to between
7 and 8 ug lysine-N (about 0.5 umoleN). However, during the derivatization of lysine with PITC, two PTC moieties attach to the lysine, each containing one nitrogen atom. Therefore, 7 to 8 ug lysine-N will yield 14 to 16 ug PTC-lysine-N (about 1 umoleN).

Considering the efficiency of the derivatization and separation method as calculated in section 5.4, it was assumed that there should be sufficient lysine-N from apoB-100 in each of the plasma samples isolated from subjects to analyse PTC-lysine samples containing 3.3 ugN in triplicate for changes in ^{15}N enrichment. At the time of the study, the c-IRMS apparatus had not been run with samples containing less than 50 ugN, which was the recommendation from the manufacturers. However, it had been suggested that the equipment could measure enrichment in smaller nitrogen samples. The aim of this section was to observe whether small changes in enrichment (as low as 0.001 APE) could be measured accurately in small samples of nitrogen (as low as 3.3 ugN) using c-IRMS.

Six stock [^{15}N]lysine solutions of differing isotopic enrichments (from 1.0 to 0.001 APE) were made. Triplicate samples were pipetted into smooth-walled tin capsules in three sets, containing either 50, 10 or 3.3 ug lysineN per sample. Samples were dried under vacuum and analysed for ^{15}N isotopic enrichment by c-IRMS. Results for the isotopic enrichment of samples are shown in table 5.2.

The results show that measured isotopic enrichment was about twice that expected. On further investigation, it was discovered that the [^{15}N]lysine used to make the stock solutions was labelled with ^{15}N on both N atoms, and so measured enrichment would in theory be twice that initially calculated. The range over which isotopic enrichment was measured was therefore 0.002 to 2.0 APE.

In the 50 ugN samples, measured enrichment correlated very closely with expected enrichment, ranging from 89 to 99 % of the theoretical maximum. There was no correlation between percentage of theoretical enrichment and the extent to which samples were enriched, suggesting that changes in ^{15}N isotopic enrichment could be accurately determined over the range of enrichment measured. In the 10 ugN samples, measured enrichment ranged from 95 to 103 % of the theoretical maximum, and in the 3.3 ugN samples, measured enrichment ranged from 84 to 91 % of the theoretical maximum. Again, there was no correlation between percentage of theoretical enrichment, and the degree to

which samples were enriched in both these sample sizes. On average, measured enrichment in the 3.3 ugN samples was 93 % that in the 50 ugN samples, and the results indicate that changes in ^{15}N enrichment as low as 0.002 APE could be measured accurately over the three nitrogen concentrations studied.

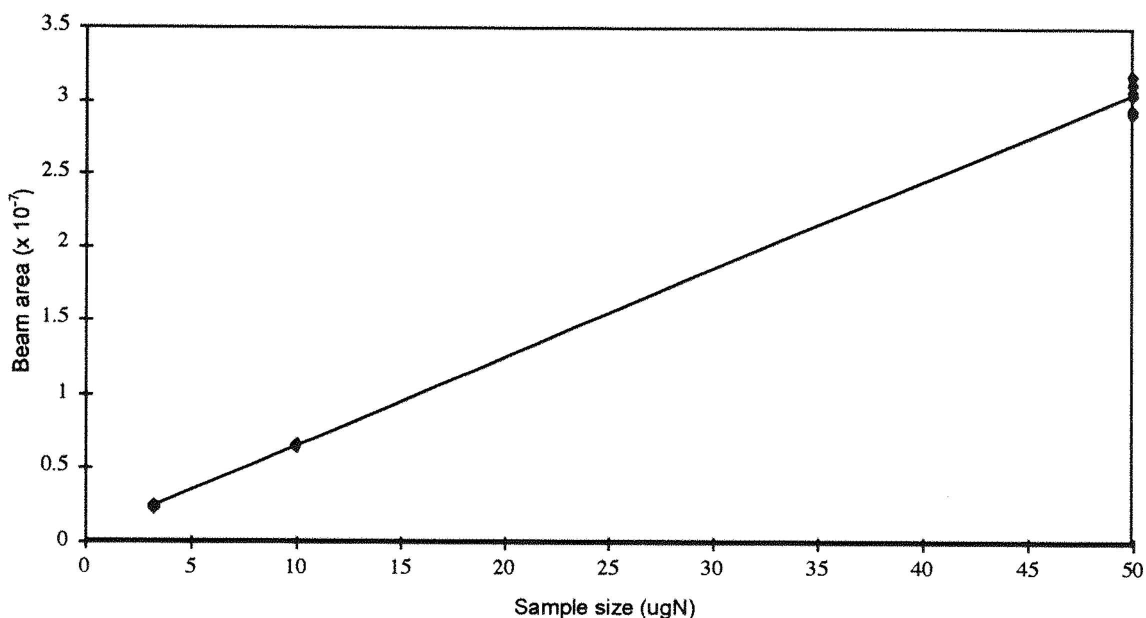
Table 5.2 Isotopic enrichment measured in six solutions of differing isotopic enrichment containing either 50, 10, or 3.3 ug lysine-N. Results are expressed as mean beam area, and the mean measured enrichment above baseline

Sample size (ug lysine-N)	*Expected APE*	*Beam Area (SD) x 10^{-8}*	*Measured APE (SD)*	*% Expected Value*
50	0	30.65 (1.27)	0.00000 (0.00005)	-
	0.002	30.35 (1.74)	0.00197 (0.00002)	99
	0.01	31.67 (0.62)	0.00964 (0.00002)	96
	0.02	31.14 (0.26)	0.01773 (0.00074)	89
	0.2	29.28 (3.03)	0.18930 (0.00087)	95
	2.0	29.10 (3.34)	1.90514 (0.00384)	95
10	0	6.58 (0.20)	0.00000 (0.00018)	-
	0.002	6.62 (0.19)	0.00206 (0.00031)	103
	0.01	6.47 (0.22)	0.00982 (0.00041)	98
	0.02	6.57 (0.09)	0.01920 (0.00002)	96
	0.2	6.66 (0.07)	0.19314 (0.00019)	97
	2.0	6.62 (0.13)	1.90194 (0.00355)	95
3.3	0	2.36 (0.09)	0.00000 (0.00033)	-
	0.002	2.43 (0.09)	0.00181 (0.00066)	91
	0.01	2.43 (0.07)	0.00834 (0.00022)	83
	0.02	2.20 (0.07)	0.01176 (0.00051)	89
	0.2	2.38 (0.14)	0.18247 (0.00614)	91
	2.0	2.36 (0.10)	1.74384 (0.05425)	87

It was interesting to note a strong positive correlation between sample size (ugN) and measured beam area ($y = 0.0598x + 0.0489$; $R^2 = 0.998$). This is displayed

graphically in figure 5.5. Variation in measured beam area between samples of the same size were low (CV <7%) suggesting that there was only small variation in the nitrogen content of samples of the same size.

Figure 5.5 Graph to show the relationship between sample size and beam area measured with c-IRMS



5.6 Measurement of ^{15}N isotopic enrichment in lysine from VLDL apoB-100 collected during a clinical trial

The previous section demonstrated that c-IRMS can accurately measure changes in ^{15}N isotopic enrichment as low as 0.002 APE in PTC-lysine samples containing 3.3 ugN. This suggests that c-IRMS is suitable for measuring changes in ^{15}N enrichment in lysine, isolated from the apoB-100 portion of plasma taken from subjects during the clinical trial detailed in chapter 4. Assuming that each blood sample collected would yield about 14 to 16 ug PTC-lysine-N, as calculated in section 5.5, and allowing for loss of sample during the derivatization and separation procedure, as calculated in section 5.4, it was assumed that it should be possible to measure changes in ^{15}N isotopic in the apoB-100 lysine of each plasma sample collected when analysed in triplicate.

The VLDL layer from each plasma sample was separated, and the apoB-100 component was precipitated and washed as described in section 2.4.6. ApoB-100 was hydrolysed under acid conditions (section 2.4.9) to release the free amino acids. PTC-amino acids were prepared as described in section 5.2. 60 ul carrier solvent were added to each sample and mixed by vortex. 20 ul aliquots were separated by HPLC in triplicate, and the PTC-lysine fractions were collected into glass vials and dried under vacuum. Eluted fractions were collected manually by visual inspection with reference to a previously run PTC-lysine standard. Samples were prepared for analysis by mass-spectrometry as described in section 5.2, and ^{15}N enrichment was determined by c-IRMS. Table 5.3 shows the results for ^{15}N isotopic abundance in PTC-lysine samples at baseline and 18 h after prime and intermittent oral dose of lactose [$^{15}N^{15}N$]ureide, and the difference in enrichment between baseline and test samples. Beam area for each set of samples is also displayed.

Measurements of both baseline and 18 h samples of apoB-100 lysine-N were successfully obtained for six of the subjects measured. ^{15}N isotopic enrichment in the 18 h samples ranged from -0.02449 to 0.01977 APE, with an average of -0.00096 (SD 0.01760) APE. This suggests that there was no significant increase in isotopic enrichment 18 h after administration of isotope compared to the baseline value in the six subjects successfully measured. Three subjects (2,3 and 4) showed a positive difference in enrichment, while three showed a negative difference (1,5 and 7). It was noted that mean ^{15}N enrichment at baseline was high, 0.38699 (SD 0.01643) APE, compared to the average natural isotopic abundance of ^{15}N (0.36630 APE). Mean beam area for all samples was 0.728×10^{8} and varied greatly between samples (CV 69 %). This is equivalent to an average sample size of 0.4 ugN according to the equation obtained from figure 5.5, and was almost ten-fold lower than the limit of detection tested in section 5.5.

Table 5.3 Isotopic enrichment in VLDL apoB-100 lysine at baseline and 18 h after a prime oral administration of lactose [$^{15}N^{15}N$]ureide in nine adult subjects consuming a diet marginally adequate in protein. Results are expressed as sample beam area, mean isotopic abundance (atoms %), and the difference in enrichment between the baseline and test sample value (APE)

Subject	*Time (hr)*	*Beam area (SD) x 10^8*	*Mean isotopic abundance (SD)*	*Enrichment (APE)*
1	0	0.468 (0.244)	0.40014 (0.02249)	
	18	1.087 (0.477)	0.37566 (0.00770)	**-0.02449**
2	0	1.150 (0.389)	0.37177 (0.00428)	
	18	0.578 (0.174)	0.38545 (0.00854)	**0.01368**
3	0	1.456 (1.453)	0.37565 (0.01158)	
	18	0.618 (0.124)	0.38375 (0.00433)	**0.00810**
4	0	0.715 (0.254)	0.38184 (0.00955)	
	18	0.344 (0.062)	0.40161 (0.00864)	**0.01977**
5	0	0.441 (0.214)	0.39766 (0.01920)	
	18	0.802 (0.371)	0.38123 (0.01429)	**-0.01643**
6	18	0.683 (0.410)	0.37066 (0.00639)	
7	0	0.668 (0.281)	0.38464 (0.00840)	
	18	0.881 (0.336)	0.37823 (0.00505)	**-0.00641**
8	0	0.519 (0.378)	0.39664 (0.02312)	
9	0	0.510 (0.175)	0.38866 (0.01230)	

Rows are shaded where both 0 and 18 h samples were not successfully measured

An interesting relationship was observed between baseline ^{15}N isotopic abundance and beam area. A graph of all baseline samples measured for the nine subjects shows that as sample size decreased, measured isotopic abundance increased, following a mono-exponential relationship (Figure 5.5; $y = 0.127994 \times e(-3.8307x) + 0.369871$). Taking this relationship into account, baseline isotopic abundance was recalculated for each sample (Table 5.4). The effect of this correction was that the mean differences in sample enrichment for

the six subjects measured tended more toward zero (range of -0.00312 to 0.00190 APE), but no significant increase in measured ^{15}N enrichment after 18 h was observed (mean enrichment of 0.00005 (SD 0.00182) APE).

Figure 5.5 Graph to show the relationship between measured baseline isotopic abundance and beam area in apoB-100 lysine samples from nine healthy adults consuming a diet marginally adequate in protein

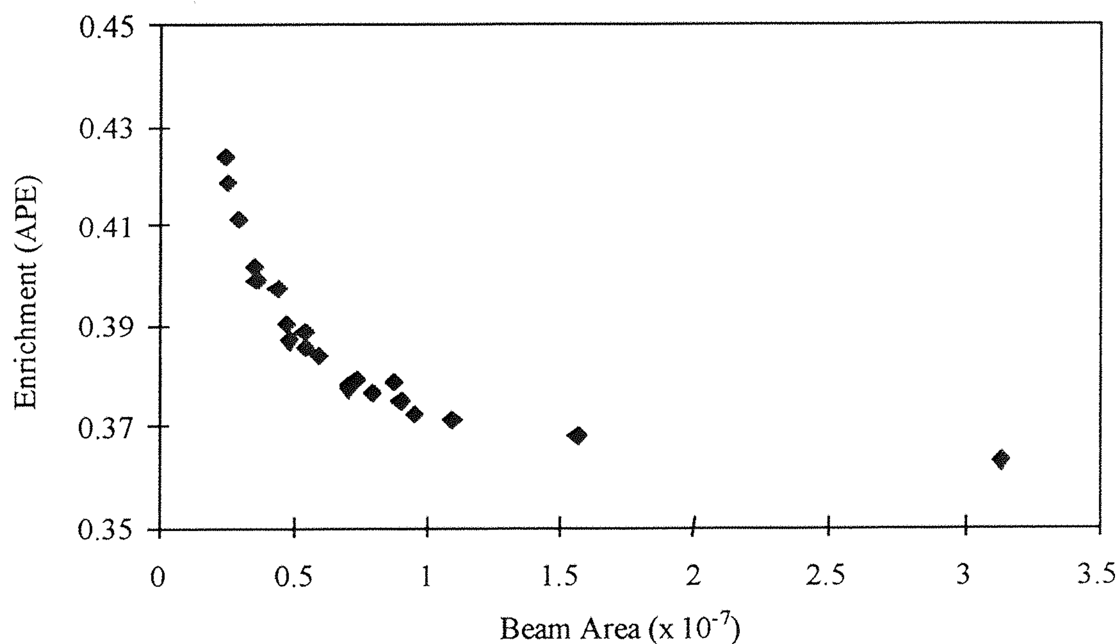


Table 5.4 Isotopic abundance in apoB-100 lysine at baseline and 18 h after a prime oral dose of lactose [$^{15}N^{15}N$]ureide in six adult subjects consuming a diet marginally adequate in protein. Results are shown as the recalculated baseline and 18 h values (atoms %), and as ^{15}N isotopic enrichment after 18 h (APE)

Subject	*Recalculated baseline*	*Isotopic abundance after 18h*	*Enrichment (APE)*
1	0.37376	0.37566	**0.00190**
2	0.38592	0.38545	**-0.00047**
3	0.38273	0.38375	**0.00102**
4	0.40473	0.40161	**-0.00312**
5	0.38162	0.38123	**-0.00039**
7	0.37689	0.37823	**0.00134**

5.7 Measured efficiency of the derivatization and separation procedures using [^{15}N]lysine

The results obtained in section 5.6 suggested that there was no significant rise in ^{15}N enrichment in apoB-100 lysine measured by c-IRMS after 18 h following prime and intermittent oral doses of lactose [$^{15}N^{15}N$]ureide in six adults consuming a diet marginally adequate in protein. However, the lysine samples analysed contained only 0.4 ugN on average, and this was about ten-fold lower than the sample size expected and the smallest sample size analysed with c-IRMS in section 5.5. Two possibilities present themselves. Either there was indeed no change in ^{15}N enrichment in apoB-100 lysine samples, or there was a change which was not detected under the conditions of the method.

Considering the small amount of lysine in the sample analysed, it seemed logical to investigate whether there was any loss of sample during the preparation of the samples. [^{3}H]lysine had already been used to estimate the efficiency of the derivatization and separation processes (section 5.4), and it was demonstrated that there was about a 40 % loss of sample in total during

processing. [^{3}H]lysine was used to follow the fate of lysine through derivatization and separation, but it does not provide any information on the fate of lysine-N. Since the enrichment of lysine with ^{15}N is of concern in this chapter, it is important to follow the nitrogen part of lysine during the preparation for mass-spectrometry. It was decided to follow the fate of lysine-N during the derivatization and separation processes using ^{15}N-labelled lysine.

Aliquots containing 3.3 ugN of previously prepared stock lysine solutions, of varying isotopic enrichment (2.0 to 0.002 APE; see section 5.5), were pipetted into tin capsules in triplicate and dried under vacuum to form *stock* samples. Further aliquots of the stock solution were derivatized in triplicate with PITC (section 5.2), transferred into tin capsules and dried to form *derivatized* samples. Separate triplicate aliquots of the stock were derivatized and then separated by HPLC (section 5.2), and the PTC-lysine fraction was collected and analysed by mass-spectrometry (the *separated* samples). The entire procedure was carried out on three separate occasions. On the first occasion, however, the procedure proceeded only until the derivatization stage, and no *separated* samples were produced. Results for the *stock* lysine samples are shown in table 5.5.

Mean beam area was 2.222×10^{-8} (CV 1%) for the first run, 1.614×10^{-8} (CV 16%) for the second, and 1.851×10^{-8} (CV 4%) for the third. The mean beam area observed in the 3.3 ugN samples analysed previously in section 5.5 (2.361×10^{-8}) was similar to the value obtained in the first run, but higher than the mean beam area in the second and third runs.

Mean measured isotopic enrichment in *stock* samples expressed as a proportion of the theoretical maximum was 76% (CV 3%) in the first run, 74% (CV 13%) in the second and 81% (CV 6%) in the third. There was no correlation between percentage of theoretical maximum and degree of sample enrichment. Isotopic enrichment, as expressed as a percentage of the theoretical maximum, was lower in all three runs than in the 3.3 ugN samples measured previously in section 5.5. (88%, CV 4%).

Table 5.5 Isotopic enrichment for six *stock* samples of differing isotopic enrichment containing 3.3 ug lysine-N, measured on three separate occasions. Results expressed as mean beam area, mean measured enrichment (APE), and % of theoretical enrichment.

Expected APE	*Run*	*Beam area x 10^{-8} (SD)*	*Measured APE (SD)*	*% Theoretical Value*
0	i	2.231 (0.147)	0 (0.00144)	
	ii	1.799 (0.057)	0 (0.00155)	
	iii	1.887 (0.412)	0 (0.00053)	
0.002	i	2.193 (0.400)	0.00146 (0.00053)	73
	ii	1.282 (0.819)	0.00125 (0.00163)	63
	iii	1.977 (0.228)	0.00160 (0.00030)	80
0.01	i	2.232 (0.194)	0.00742 (0.00054)	74
	ii	1.288 (0.752)	0.00643 (0.00329)	64
	iii	1.852 (0.090)	0.00732 (0.00099)	73
0.02	i	2.225 (0.180)	0.01560 (0.00153)	78
	ii	1.821 (0.095)	0.01495 (0.00353)	75
	iii	1.836 (0.121)	0.01641 (0.00068)	82
0.2	i	2.226 (0.155)	0.15768 (0.01347)	79
	ii	1.749 (0.012)	0.16722 (0.00059)	84
	iii	1.775 (0.060)	0.17191 (0.00364)	86
2.0	i	2.225 (0.330)	1.53251 (0.20625)	77
	ii	1.744 (0.172)	1.63470 (0.05274)	82
	iii	1.776 (0.138)	1.66037 (0.05389)	83

Results for the *derivatized* PTC-lysine samples are shown in table 5.6. Mean beam area was 5.417×10^{-8} (CV 20%) for the first run, 4.865×10^{-8} (CV 20%) for the second, and 4.549×10^{-8} (CV 10%) for the third. On average, mean beam area over all three *derivatized* runs was 2.6 times higher than the average beam area over all three *stock* runs, and this corresponds to the increase in sample nitrogen as a result of the derivatization process.

Table 5.6 Isotopic enrichment for six *derivatized* samples of differing isotopic enrichment containing 3.3 ug lysine-N, measured on three separate occasions. Results expressed as mean beam area, mean measured enrichment (APE), and % of theoretical enrichment.

Expected APE	*Run*	*Beam area x10^{-8} (SD)*	*Measured APE (SD)*	*% Theoretical Value*
0	i	6.083 (2.115)	0 (0.00009)	
	ii	4.881 (0.121)	0 (0.00108)	
	iii	4.743 (0.904)	0 (0.00686)	
0.002	i	3.753 (0.224)	0.00091 (0.00043)	91
	ii	4.435 (0.247)	-0.00097 (0.01279)	
	iii	4.528 (0.168)	-0.01041 (0.00164)	
0.01	i	6.856 (3.315)	0.00304 (0.00129)	61
	ii	4.542 (0.203)	0.00089 (0.00130)	18
	iii	3.946 (0.614)	-0.01032 (0.00181)	
0.02	i	5.240 (0.620)	0.00676 (0.00076)	68
	ii	4.239 (0.263)	0.00350 (0.00085)	35
	iii	4.553 (0.635)	-0.00785 (0.00164)	
0.2	i	4.862 (0.091)	0.07131 (0.00183)	71
	ii	6.830 (2.902)	0.05711 (0.00416)	57
	iii	5.237 (1.437)	0.03164 (0.01636)	32
2.0	i	5.719 (0.692)	0.62405 (0.05954)	62
	ii	4.261 (0.272)	0.57542 (0.05117)	58
	iii	4.290 (1.017)	0.53491 (0.04011)	53

Rows are shaded where a negative enrichment value was obtained

Mean measured isotopic enrichment in the first run of the *derivatized* samples was 91 % that of the mean *stock* sample enrichment , and 71% (CoV 17%) of the theoretical maximum the first run (the calculation takes into account the dilution of isotope during the derivatization process). In the second and third runs, value for enrichment were negative in some of the samples analysed. Overall, isotopic

enrichment in the *derivatized* samples was 71% of the mean *stock* sample enrichment (discounting negative values).

Results for the *separated* PTC-lysine samples are shown in table 5.7. Mean beam area was 2.924×10^{-8} (CV 22%) for the first run and 8.263×10^{-8} (CV 21%) for the second, suggesting that, on average, about half the sample size recorded in the *derivatized* samples was lost in the first run, whereas sample size appeared to double in the second run.

Table 5.7 Isotopic enrichment for six *separated* samples of differing isotopic enrichment containing 3.3 ug lysine-N, measured on two separate occasions. Results expressed as mean beam area, mean measured enrichment (APE), and % of theoretical enrichment.

Expected APE	*Run*	*Beam area x 10^{-8} (SD)*	*Measured APE (SD)*	*% Theoretical Value*
0	i	3.357 (1.117)	0 (0.00498)	
	ii	10.734 (1.099)	0 (0.00257)	
0.002	i	2.528 (0.600)	-0.00283 (0.00334)	
	ii	9.216 (1.590)	-0.00160 (0.00273)	
0.01	i	2.344 (0.177)	-0.00256 (0.00245)	
	ii	8.933 (0.287)	-0.00063 (0.00161)	
0.02	i	2.381 (0.683)	-0.00091 (0.00191)	
	ii	8.224 (1.504)	0.00112 (0.00124)	11
0.2	i	2.966 (0.797)	0.02719 (0.01242)	27
	ii	6.009 (2.216)	0.02956 (0.02071)	30
2.0	i	3.971 (1.585)	0.28755 (0.12607)	29
	ii	6.464 (2.000)	0.22952 (0.05777)	23

Rows are shaded where a negative enrichment value was obtained

Values for the mean enrichment were negative in both runs for samples with a low expected enrichment (0.02 APE and below). Excluding negative values,

overall mean sample enrichment was only 24 % (CoV 32%) of the theoretical maximum, and 29 % of the mean *stock* sample enrichment.

5.8 Discussion

This chapter has been divided into six sections, describing:

a methods to derivatize amino acids with PITC, separate the resultant PTC-amino acids using HPLC, and analyse sample for changes in ^{15}N enrichment using c-IRMS

b the separation and identification of PTC-lysine from a mixture of 20 PTC-amino acids

c an investigation into the loss of lysine during the derivatization and separation procedures, using [^{3}H]lysine

d analysis of ^{15}N enrichment in PTC-lysine samples of various size and isotopic enrichment using c-IRMS

e analysis of ^{15}N enrichment in lysine isolated from VLDL apoB-100 samples taken during a clinical trial

f an investigation into the loss of lysine-N during the derivatization and separation procedures, using [^{15}N]lysine.

A method was devised to form PTC-amino acids by reacting amino acids with PITC. Derivatization of amino acids with PITC is typically an automated process when combined with analytical HPLC, but the quantities of lysine required in this chapter for the subsequent analysis of changes in ^{15}N enrichment using c-IRMS meant that the procedure had to be scaled up, and a manual method was developed. The derivatization process is relatively rapid, the reaction of PITC with amino acids being complete within 10 to 20 min. Some authors have suggested

that PTC-amino acid derivatives can only be successfully produced under totally oxygen free-conditions, while others disagree (Heinrikson and Meredith, 1984). It had been observed during the initial stages of method development that large unknown peaks often eluted during the separation stage. Steps were taken to minimise contact of PITC and the resultant PTC-amino acids with the air, by derivatizing samples under a stream of nitrogen gas and using a fresh ampoule of PITC each time derivatizing reagent was made. As a result, the unknown peaks disappeared. All samples were kept at -20°C before analysis, since PTC-amino acids are reported to degrade quickly at room temperature.

PTC-lysine was separated from a mixture of 20 PTC-amino acids using HPLC. PTC-lysine eluted after about 36 minutes, but each separation run lasted for 75 minutes to prevent any cross-contamination between runs. For example, a peak consistently eluted at about 71 min each time a sample was run, which probably corresponded to some unknown PITC adduct. PTC-lysine was the last PTC-amino acid to be eluted, and the corresponding peak was well resolved from the others. Additionally, the di-derivatisation of lysine with PITC made for easy detection of the PTC-lysine peak at 254 nm. Excluding the peak corresponding to PTC-lysine, only 14 other peaks corresponding to 19 PTC-amino acids were observed (Figures 5.2 and 5.3). This is possibly due to co-elution of some PTC-amino acids, but this had no effect on the resolution of the PTC-lysine peak. Elution times for PTC-lysine were similar between runs conducted on different days. Small differences in elution time were probably due to changes in ambient temperature, pressure, and buffer concentration, and a PTC-lysine standard was run at the beginning of each day to calibrate the HPLC equipment . Overall, the derivatization and separation methods developed allowed for a relatively straightforward, quick and reliable procedure for isolating PTC-lysine from a mixture of 20 PTC-amino acids.

The efficiency of the derivatization and separation procedures was assessed by following the fate of [^{3}H]lysine through these two stages of the method. The results suggested that there was an overall loss of about 40% of lysine during the derivatization and separation stages. Assuming that the plasma samples isolated from subjects during the lactose [$^{15}N^{15}N$]ureide study detailed in chapter

4 would contain about 14-16 ug PTC-lysine-N, the loss of lysine during separation and derivatization calculated from the loss of [^{3}H]lysine meant that about 10 ug PTC-lysine-N could be isolated from each plasma sample. This would allow for the measurement of changes in ^{15}N enrichment using c-IRMS in samples of 3.3 ugN measured in triplicate. The capacity for c-IRMS to measure changes in ^{15}N enrichment in samples containing 3.3 ugN had to be determined. Lysine standards of varying nitrogen content (50, 10 and 3.3 ugN per sample) and ^{15}N enrichment (2.0 to 0.002 APE) were prepared and analysed for ^{15}N enrichment by c-IRMS. The results suggested that the c-IRMS equipment could measure changes in ^{15}N enrichment as low as 0.002 APE in samples containing 3.3 ugN within 90 % of the enrichment measured in samples containing 50 ugN.

The finding that c-IRMS could accurately measure changes in ^{15}N enrichment in samples containing only 3.3 ugN was significant because it suggested for the first time that it should be possible to reliably measure small changes in enrichment (as low as 0.002 APE) in the lysine samples isolated from apoB-100 during the clinical trial described in chapter 4. The test samples were prepared, the lysine part separated and analysed for changes in ^{15}N enrichment. No significant increase in lysine ^{15}N enrichment was observed between the baseline samples and those taken 18 h after a prime and intermittent doses of lactose [$^{15}N^{15}N$]ureide in the six subjects successfully measured. However, two interesting points were observed. The first relates to the sample size.
A relationship between measured beam area and expected sample size had been observed in section 5.5, and this allowed for a calculation of the size of PTC-lysine samples isolated from apoB-100 lysine. Average sample size was calculated to be about 0.4 ugN, almost ten-times lower than that expected and similarly nearly ten-times lower than the tested limit of the c-IRMS apparatus. Further, an interesting relationship was observed between measured beam area in the baseline PTC-lysine samples and measured isotopic enrichment, such that measured enrichment increased as beam area decreased. Values for changes in PTC-lysine ^{15}N enrichment were further corrected for this observed phenomenon, although this only had the effect of decreasing the range of differences in enrichment observed between baseline and 18 h samples.

Since the PTC-lysine samples isolated from plasma apoB-100 were over ten-times smaller than had been anticipated, it was appropriate to further investigate the derivatization and separation procedures used to prepare PTC-lysine. [^{3}H]lysine had been used to provide information on the loss of total lysine during these two stages. It was also desirable to specifically follow the fate of the nitrogen part of lysine, as well as lysine as a whole. [^{15}N]lysine standards containing 3.3 ugN and a range of isotopic enrichments were derivatized, separated and analysed on three occasions. After the first derivatization run, sample enrichment did not decrease significantly, and was over 90% that reported in the stock samples. However, in the next two runs, measured enrichment fell, and the average enrichment was calculated as 71 % of the mean *stock* enrichment. There was a big decrease in enrichment expressed as a proportion of the stock enrichment after separation and analysis of samples. Table 5.8 shows a comparison of the relative loss of enrichment after sample derivatization and separation compared to the respective stock value when using either [^{3}H]lysine or [^{15}N]lysine.

Table 5.8 Comparison of the loss of either [^{3}H]lysine or [^{15}N]lysine during the derivatisation and separation procedures. Results are expressed as a percentage of the stock value.

	[^{3}H]Lysine		*[^{15}N]Lysine*	
	MEAN	CV	MEAN	CV
Stock	100	3	100	9
Derivatized	75	26	71	38
Separated	61	18	29	31

This comparison indicates that on average, the loss of total lysine and lysine-^{15}N were similar after samples had been derivatized compared to the stock value. However, after separation with HPLC and analysis of enrichment, more lysine-^{15}N was lost than total lysine. This suggests that the loss of ^{15}N enrichment during separation is higher than can be accounted for solely by the loss of total lysine. It is possible that during the separation of PTC-lysine using HPLC, some sample fractionation occurred, such that [^{15}N]lysine eluted at a slightly different time to

unlabelled lysine. During collection of the PTC-lysine peak, the amount of eluent collected may not have been sufficient to include all the [^{15}N]lysine, and a small variation in the total amount of the lysine peak collected could result in a large variation in the total amount of [^{15}N]lysine collected. Measured beam area for the derivatized samples increased by an average of 2.6 times compared to the stock samples, corresponding to the increase in sample nitrogen incurred during derivatization. This would tend to suggest that no actual N was lost during the derivatization process, although there was a fall in enrichment. Similarly, changes in mean sample size after separation calculated from the beam area did not in any way resemble changes in ^{15}N enrichment after separation. Taking these results together, this would suggest that there is no apparent correlation between the fate of either total lysine, total lysine-N, or total lysine-^{15}N through the derivatization and separation procedures.

In summary, methods were devised to separate lysine from a mixture of 20 amino acids using HPLC, and to accurately measure changes in ^{15}N enrichment as low 0.002 APE in lysine samples containing 3.3 ugN using c-IRMS. Lysine samples were isolated from the VLDL apoB-100 fraction of plasma samples taken from subjects consuming a diet marginally adequate in protein during a clinical trial. Although no significant increase in lysine-N enrichment was detected after 18 h following a prime and intermittent oral doses of lactose [$^{15}N^{15}N$]ureide, it is not possible to say at this time whether these results represent the true changes in enrichment, since the mean sample size tested was nearly ten-times lower than the smallest sample sizes tested using the c-IRMS apparatus. Additionally, on further investigation there appeared to be substantial loss of ^{15}N enrichment in samples of [^{15}N]lysine during the HPLC separation procedure, possibly due to sample fractionation. A clear distinction was made between the fates of lysine, lysine-N, and lysine-^{15}N during the derivatization and separation procedures. The method offers a potential valuable means to measure small changes in ^{15}N enrichment in small samples of biological material, such as apoB-100 lysine, and it would seem to be worthwhile to undertake further investigations to minimise the loss of ^{15}N enrichment during the separation procedure, and further test the limits to which c-IRMS can accurately detect changes in ^{15}N enrichment.

Appendix 5A HPLC Information

Coupling Reagent

2 ml ethanol; 2 ml deionised water; 1 ml triethylacetic acid

Derivatization Reagent

7 ml ethanol; 1 ml deionised water; 1 ml triethyacetic acid;
1 ml phenylisothiocyanate

Both coupling and derivatisation reagents were made up fresh every day. PITC is extremely sensitive to oxygen and temperature. Therefore, it was purchased in 1 ml ampoules sealed under argon, and stored at 4 °C. A fresh ampoule was used in each set of reactions. The derivatisation reagent was made up under a constant stream of nitrogen gas, and each sample tube was evacuated with nitrogen after the reagent had been added to minimise oxygen interference.

Equipment

Column - Dynamax 60A reverse-phase C18 column (Ranin Instruments Co., MA, USA)
Pumps - Gilson 305 and 307 with 10SC pump head
Computer - 486 PC running Gilson 714 controlling software
Interface - Gilson 506C system interface module
Mixer - Gilson 811C dynamic mixer
Detector - Milton Roy SM4000 programmable wavelength detector
Autoinjector - Gilson 231 sample injector and Gilson 401 dilutor
Collector - Gilson FC 205 fraction collector

Solvents

A - 0.14M sodium acetate containing 0.5 % TEA at pH 6.15
B - 60 % acetonitrile (Rathburns, Edinburgh, UK)
Carrier solvent - 10 % B in A
Solvents degassed by sonication under vacuum

Solvent Gradient

Time (min)	*Flow (ml/min)*	*%B*
0	2.0	10
5	2.0	10
45	2.0	90
46	4.0	100
72	4.0	100
74.5	2.0	10
75	2.0	10

CHAPTER 6

General Discussion

The purpose of this final chapter is four-fold. First, to restate the aim of the thesis within the relevant scientific background. Second, to summarise the experimental work. Third, to discuss the implications of the work, and finally, to present the conclusions of the thesis.

6.1 Aim and background

Since the discovery over 200 years ago that a dietary source of nitrogen is essential for life, there has been a continuing need to understand human protein, amino acid and nitrogen requirements. Criticism of the importance of this area of scientific research has been recently met by Fuller and Garlick (1994), who have asked, *'Is it acceptable that we humans, with our vast collection of scientific knowledge, should enter the twenty-first century still ignorant of something as basic as what our bodies require for sustenance?'*.

The increase in methods available to study human nitrogen metabolism have allowed for a further understanding of the requirement for protein, amino acids, and nitrogen. Initial studies of nitrogen balance identified that there was a continual need for protein in the diet to maintain life. Even when the dietary intake was zero, nitrogen was lost from the body in the excreta. Such experiments led to proposals for a daily dietary protein requirement which was met for each individual at nitrogen balance, when intake of nitrogen in the diet, and excretion of nitrogen in urine and stool, were equal. However, observations that the body can maintain nitrogen balance over a wide range of protein intakes led to equally wide-ranging recommendations for dietary protein intake, initially confounded by Leibig's hypothesis that protein was the sole source of energy for muscular work.

The identification and purification of amino acids in the early part of this century added a new level of complexity to the issue of requirements. Amino acids were classified as either 'essential' or 'non-essential' in the diet, denoting the capacity of the body to synthesize them to meet the body's needs, and dietary requirement values for individual amino acids were published by Rose (1957). However, attaining nitrogen balance was still used as the criterion of nutritional adequacy, and the mechanisms by which the body can adapt to a variety of protein intakes was still not clear.

The current FAO/WHO/UNU (1985) recommendations for amino acid requirements are based on studies of nitrogen balance. The use of stable isotopes in biological research has provided a powerful tool with which to further investigate nitrogen metabolism. The metabolic fate of proteins, individual amino acids, and other organic nitrogenous substances, such as urea and ammonia, was made possible. This has led to further insights into the study of protein, amino acid, and nitrogen requirements, and their metabolic control. Complex studies using ^{13}C-labelled amino acids have been conducted, resulting in a challenge to current FAO/WHO/UNU (1985) recommendations for amino acid requirements. Subsequent proposals for dietary amino acid requirements have been outlined by Young and colleagues (1989), and are significantly higher than those currently recommended. Consequently the

assumptions around which these proposals are based need to be assessed critically. One criticism of the new proposals is that the theoretical model around which they are based takes no account the phenomenon of colonic urea salvage (Jackson, 1995). Urea, classically viewed as the nitrogenous 'end-product' of amino acid metabolism, was shown to be hydrolysed through the actions of the colonic microflora in normal adults nearly 40 years ago (Walser and Bodenlos, 1959), the nitrogenous part being available for further body metabolism. The subsequent study of urea kinetics, especially by Jackson and co-workers, has shown that the intensity of urea hydrolysis increases as dietary protein intake falls (Langran *et al*, 1992; Bundy *et al*, 1993; Meakins *et al*, 1996), leading to the suggestion that the process has a functional significance. However, direct evidence to support the physiological importance of this process has not been presented, and has only been inferred from studies of urea kinetics.

In an attempt to resolve the important debate concerning the functional significance of urea salvage, an obvious route is a direct quantification of the fate of salvaged urea-N. This involves presenting a known dose of ^{15}N-labelled urea directly to the site of urea salvage, the colon, but previous methodology has been limited to an invasive method not applicable to normal adults (Moran and Jackson, 1990*a,b*). *The aim of the thesis was to develop a novel method which could be used to non-invasively quantify the fate of urea-N salvaged in the colon of normal adults.*

The form in which salvaged urea-N is made available to the body is another important subject of continuing debate, and there is a suggestion that essential amino acids synthesised *de novo* by the colonic bacteria may be available to the host in functionally significant amounts. This raises important questions concerning the classification of amino acids as 'essential or 'non-essential', and the theoretical framework around which dietary requirements for amino acids have been calculated (Millward *et al*, 1991). Previously, a quantification of the possible utilisation of amino acids produced through the action of the colonic microflora has not been possible due to methodological limitations.

A further part of the thesis was to develop a method to quantify the potential utilisation of de novo formed lysine by the body.

6.2 Experimental studies

In order to quantify the fate of salvaged urea-N using a stable isotope methodology, a known dose of label must be presented directly to the colon, the site of urea hydrolysis. In studies of urea kinetics, the amount of orally or intravenously administered [$^{15}N^{15}N$]urea presented to the colon cannot be known directly. Direct presentation of [$^{15}N^{15}N$]urea to the colon has been achieved through a colonoscope (Moran and Jackson, 1990*a*,*b*), but this invasive technique has only limited application. Lactose-ureide, isotopically labelled on the urea part, has been previously used as a non-invasive marker of ileocoecal transit time (Heine *et al*, 1995), and provides a potential means to non-invasively present ^{15}N label directly to the colon. This assumes that labelled lactose-ureide is not metabolised in the upper gut, and therefore passes down to the colon intact before bacterial fermentation and hydrolysis release the labelled nitrogen. The body is reportedly unable to break the bond between lactose and urea (Heine *et al*, 1995), whereas recently direct evidence has shown that a number of microbial species resident the colon can (Wutzke *et al*, personal communication).

The experimental work undertaken to address the aim of the thesis can be divided into three parts. The first stage was to synthesize two lactose-ureides, isotopically labelled with either [^{13}C] or [$^{15}N^{15}N$] on the urea part. Labelled lactose-ureides are not commercially available, and custom synthesis is costly. Following the method of Hofmann (1932), lactose [^{13}C]ureide and lactose [$^{15}N^{15}N$]ureide were both synthesized. The integrity of these compounds was confirmed using a number of laboratory techniques, and further analysis revealed that there was minimal contamination from unreacted reagents and other compounds. The yield of lactose-ureides was comparable to what had been previously reported, and it was concluded that both lactose [^{13}C]ureide and

lactose [$^{15}N^{15}N$]ureide had both been successfully synthesised to a high degree of purity.

The second stage was to determine the suitability of lactose-ureide as a non-invasive carrier of isotopic label directly to the colon. Previous evidence at the time of the study was limited to one unpublished report (now presented in Heine *et al*, 1995) which found that after oral administration of a single dose of lactose [^{13}C]ureide in one adult subject, breath $^{13}CO_2$ enrichment increased after 6 h, peaked at 8h following the dose, and then tended toward baseline after 24 h. To provide further data on lactose-ureide metabolism, a clinical trial was conducted. Nine adult subjects were provided with a diet providing about 36g protein/d on average, and the excretion of a single oral dose of lactose [^{13}C]ureide as $^{13}CO_2$ on the breath was measured over 48 h. The results showed that there was very little excretion of label before 6 h, after which excretion rose rapidly and peaked at between 11 and 18 h after dose. Excretion then fell slowly, but was sometimes accompanied by a second smaller peak in excretion which is possibly due to release of label which had been trapped in a compartment of the body bicarbonate pool. By 48 h after dose, breath $^{13}CO_2$ enrichment approached baseline in all subjects. Most of the lactose [^{13}C]ureide, about 80% on average, was excreted as breath $^{13}CO_2$ breath over the 48 h of measurement. This value is significantly higher than that reported by Heine and colleagues (1995), who observed an average recovery of 37 % of a single dose of lactose [^{13}C]ureide in 14 adult subjects, but $^{13}CO_2$ was only measured for between 16 and 24 h after dose, and so did not take account of label excreted after this time. It is concluded that lactose-ureide is not metabolised in the upper gut to any significant extent, but undergoes rapid fermentation and hydrolysis once it reaches the colon, with most of the label following this fate over 48 h. Therefore, lactose-ureide was deemed to be a suitable vehicle for non-invasively delivering a known dose of ^{15}N to the colon intact.

The third stage of experimental work was to use an oral prime and intermittent dose regimen of lactose [$^{15}N^{15}N$]ureide to quantify the fate of salvaged urea-N in the nine subjects under the same study conditions. The data obtained demonstrate a number of points. A proportion of label was excreted into the

urine as [^{15}N^{15}N]urea, and this excretion reached isotopic plateau in all subjects after 6 h following the prime dose. This is taken to represent lactose [^{15}N^{15}N]ureide which is fermented in the colon, with the [^{15}N^{15}N]urea part then being directly absorbed and excreted from the body. On average, about 5 % of the rate of administration of label was excreted as [^{15}N^{15}N]urea, and variability was low between subjects. The data provide further evidence to support the notion that the colon is permeable to the intact urea molecule, and the proportion of label excreted in this form is similar to what was reported when [^{15}N^{15}N]urea was placed in the colon directly through a colonoscope (Moran and Jackson, 1990*a*)

A direct quantification of the rate of excretion of label as urinary [^{14}N^{15}N]urea was possible in four of the nine subjects who achieved isotopic plateau in the urinary [^{14}N^{15}N]urea fraction over the period of measurement. The data demonstrate that about 30 % of the rate of label administration was excreted as urinary [^{14}N^{15}N]urea in these subjects. This is of importance since it has previously been assumed that urea-N is salvaged primarily in the form of ammonium ions, which can then be incorporated into the body nitrogen pool through transamination pathways. This route has been criticised as providing no more than a futile recycling of urea-N (Jackson *et al*, 1982). However, recent work has suggested that if urea-N was salvaged and presented to the liver only as ammonium ions, about 70 % would be immediately reconverted to urea (see Waterlow, 1996). The present data support those of Jackson and colleagues, who have demonstrated through studies of urea kinetics in adults that only about 10 - 20 % salvaged urea-N is reconverted to urea on average (Hibbert *et al*, 1992; Langran *et al*, 1992; Hibbert *et al*, 1995; Meakins and Jackson, 1996) occasionally up to 40 % (Jackson *et al*, 1984; Hibbert *et al*, 1992). The present data strengthen the hypothesis that urea-N is salvaged in forms other than ammonium ions, possibly as amino acids.

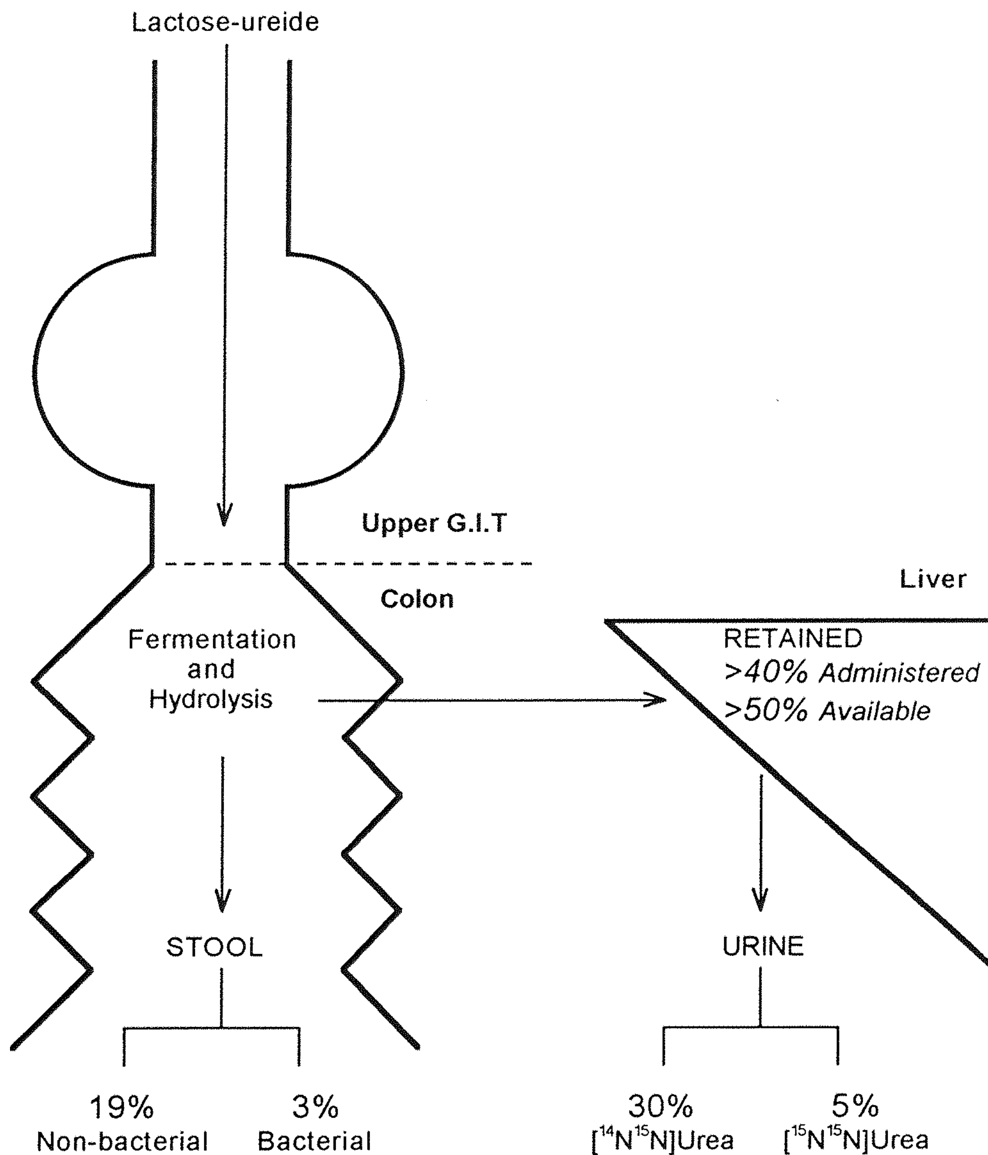
22 % of the total dose of lactose [^{15}N^{15}N]ureide was excreted in the stool on average in the nine subjects over the period of collection. This value is higher than what has previously been reported when [^{15}N^{15}N]urea was instilled directly into the colon through a colonoscope (Moran and Jackson, 1990*a*), and this is

taken to represent a slower overall fermentation and hydrolysis rate of lactose-ureide compared with urea. About 15 % of the label in stool was associated with the bacterial fraction on average (A Hounslow, personal communication), and the remaining 85 % is assumed to be in the form of unfermented lactose [$^{15}N^{15}N$]ureide, which allows a distinction to be made between the total dose of label *administered*, and the dose acted upon by the bacteria and then *available* to the body. Using this information, it is also possible to correct for the recovery of $^{13}CO_2$ on the breath over 48 h following a single oral dose of lactose [^{13}C]ureide, leading to the suggestion that, on average, over 90 % of the dose of lactose [^{13}C]ureide acted upon by the bacteria was excreted as $^{13}CO_2$ in the breath.

The form in which label is associated with the bacterial fraction of the stool has recently been investigated (Gibson *et al*, 1997*b*). Significant levels of ^{15}N enrichment were found in four amino acids isolated from bacterial hydrolysates in most subjects, including lysine. This indicates that the colonic bacteria are able to incorporate ^{15}N from lactose [$^{15}N^{15}N$]ureide into amino acids, including lysine, and taken together with the observations of Waterlow (1996), this provides further evidence to suggest that amino acids synthesized *de novo* by the colonic bacteria are made available to the body.

This is the first time that the fate of urea-N salvaged in the colon of normal adults has been quantified using a non-invasive technique. The findings of the present studies are summarised in figure 6.1, and lead to the conclusion that, of the lactose [$^{15}N^{15}N$]ureide acted upon by the bacteria and available to the body, *about half* is retained, presumably in some metabolically useful form.

Figure 6.1 The metabolism of orally administered lactose [$^{15}N^{15}N$]ureide in adults consuming a diet marginally adequate in protein.



In addition to addressing the aim of the thesis, further work was undertaken to develop a method to separate lysine from apoB-100 hydrolysates, which were isolated from blood samples taken during the clinical trial, in a quantity sufficient to accurately measure ^{15}N enrichment with mass spectrometry. The purpose was to directly address the question - is there a functional significance of lysine synthesized *de novo* by the colonic bacteria to the host? Current ion-exchange separation of protein hydrolysates, as pioneered by Moore and colleagues (1958), is technically complex and requires 24 h for complete amino acid separation using automatic recording equipment. This final part of the work was

approached by combining three technologies: PITC derivatization of amino acids to form PTC-adducts, as used in classical Edman degradation of proteins; HPLC of PTC-derivatised amino acids as developed by Bidlingmeyer and colleagues (1982); and analysis of ^{15}N isotopic enrichment using the new technique of c-IRMS. The derivatization step was successfully performed manually, contrary to previous reports which have stressed the use of an automated system to avoid any contact of the reagents with oxygen in the air (Heinrikson and Meredith, 1984; Walker and Mills, 1995). The HPLC stage was scaled up from an analytical to a semi-preparative method in order that sufficient lysine could be separated for further analysis of isotopic enrichment with mass spectrometry. Eluted PTC-lysine peaks were well resolved from other PTC-amino acids, easy to identify, and had consistent elution times. Separation was complete within 75 min, ensuring no cross-contamination between consecutive runs. At mass spectrometry, a difference in enrichment of 0.002 APE in samples of PTC-lysine as small as 3.3 ugN could be measured accurately as compared with the usual operating sample size of 50 ugN, allowing for the possibility to measure changes in isotopic enrichment in small amounts of biological material. No significant increase in ^{15}N enrichment was observed in samples of lysine isolated from plasma apoB-100 in subjects 18 h following administration of lactose [$^{15}N^{15}N$]ureide, but it is not possible at present to conclude whether this is representative of the truth, since the amount of nitrogen in the tested samples was about 10 times lower than had been accounted for and tested for using the mass spectrometry apparatus. Nevertheless, two interesting correlations were observed, one between sample size and beam area, and another between measured baseline enrichment and sample size. A distinction was also made between the fate of lysine, lysine-N, and lysine-^{15}N during the derivatization and separation procedures. The methodology, with further refinements, has application for separating lysine, and potentially other amino acids, from protein hydrolysates taken during isotope labelling studies, in order to directly address the potential functional significance of amino acids synthesized *de novo* by the colonic bacteria.

6.3 Implications of the work

The whole research area of adult nitrogen requirements has been confounded by lack of consistency over the definition of 'requirement', and therefore the theoretical models around which assumptions are made. This is illustrated in the form in which nitrogen is consumed in the diet. Originally, researchers studied the *dietary* requirement for *protein*, using nitrogen balance as the criterion of adequacy. This later developed into the *dietary* requirement for specific *amino acids*, and their classification as 'essential or 'non-essential' in the diet. The ability of the body to adapt to a range of nitrogen intakes has also led to investigation of the form, or 'quality', of nitrogen consumed, and how this relates to a broader *dietary* requirement for *nitrogen*. It is therefore important that researchers are clear in their definition of requirement.

Concentrating on amino acid requirements, definitions again need clarity. Millward and colleagues have suggested the idea of a *minimum dietary* requirement for amino acids, which allows nitrogen balance, and an *optimal dietary* requirement, which confers additional benefit to the individuals such as improved immune response and skeletal muscle function, and decreased disease morbidity (Millward and Rivers, 1988). These outcome measures, however, are difficult to quantify. Jackson has also attempted to clarify some of the definitions, by distinguishing between a *dietary* requirement and a *physiological* or *metabolic* requirement for amino acids. He suggests that the *dietary* requirement for protein, and consequently for amino acids, is that which is needed to balance the shortfall between the *metabolic* requirement for amino acids, and the endogenous supply. The endogenous supply comprises of amino acids from protein degradation and *de novo* synthesis. The increasing evidence that *de novo* amino acids, both dispensable and indispensable, may be obtained through the salvage of urea-N in the colon, needs attention with respect to the assumptions upon which calculations for dietary amino acids requirement values are based. Two recent reports provide further evidence which support this hypothesis. The first is the cloning and characterisation of a vasopressin-regulated urea transporter in the colon of rabbits (You *et al*, 1993). It has classically been assumed that urea freely diffuses across membranes in

the body, but increasing evidence has shown the present of regulated transporters in the kidney of rabbits and rats (You *et al*, 1993; Smith *et al* 1995), and in human erythrocytes (Olives *et al*, 1994). The presence of a transporter in the colon is certainly of some functional significance, and may be involved in the control of urea salvage under different metabolic conditions. The second is an intestinal peptide transporter which has been cloned, and found to be expressed in the human colon (Dantzig *et al*, 1994). This presence of this transporter, HPT-1, in the colon would strongly suggest that it has some functional significance. Both of these findings further dispel the notion that the colon is merely a storage organ which only reabsorbs salt and water. Additionally, recent work (Yeboah *et al*, 1996; Gibson *et al*, 1997*a*) has observed lysine, plus some other non-essential amino acids, significantly enriched with ^{15}N in the urine of infants receiving treatment for severe protein-energy malnutrition, following prime and intermittent oral doses of [$^{15}N^{15}N$]urea over 36 h. Finally, based on ^{15}N-lysine enrichment in the stool and urine of nine adults 48 h after oral doses of lactose [$^{15}N^{15}N$]ureide, Gibson and colleagues (1997*b*) have recently calculated that the rate of lysine appearance across the colon equates to approximately 29 mg/kg/d, more than twice the current FAO/WHO/UNU (1985) recommended daily intake for lysine in adults. These findings further support the role of urea salvage as a biologically important source of *de novo* synthesized essential amino acids.

The studies of Young and colleagues using ^{13}C-labelled amino acids to assess adult amino acid requirements have been carefully conducted, and provide a wealth of data which agree closely with the theoretical model which they have proposed. However, the fact that the model takes no account of the potential supply of amino acids synthesised *de novo* in the colon, despite of the evidence which has accumulated to the contrary, makes increasingly unjustifiable Young's viewpoint that it is merely an 'extravagant claim' (El-Khoury *et al*, 1994*a*). Since the economic import of this claim would appear to be as great as the conceptual one, addressing the problem directly would seem crucial. It is hoped that the methodology developed and experimental results obtained in the thesis have helped in this endeavour, and will continue to do so.

6.4 Conclusions

Three specific aims were set out in the introduction to the thesis. All three have been addressed.

a Lactose [^{13}C]ureide and lactose[$^{15}N^{15}N$]ureide have both been successfully synthesized and analysed for purity.

b Lactose [^{13}C]ureide has been used to show that lactose-ureide is a suitable vehicle to non-invasively deliver of known dose of isotope directly to the site of urea salvage, the colon, where it is then fermented and hydrolysed by the resident bacteria.

c Lactose [$^{15}N^{15}N$]ureide has been used for the first time to quantify the fate of urea-N salvaged the colon, demonstrating that over half of the dose available to the body was retained in adults consuming a diet marginally adequate in protein.

Additionally, a further new methodology has been developed to isolate lysine from small amounts of biological material in a sufficient quantity to measure ^{15}N isotopic enrichment with c-IRMS.

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