UNIVERSITY OF SOUTHAMPTON

THE INFLUENCE OF DIETARY NITROGEN INTAKE ON UREA-NITROGEN SALVAGE IN THE COLON

TRACEY SUZANNE MEAKINS

THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF HUMAN NUTRITION

FACULTY OF MEDICINE

FEBRUARY 1996

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE
HUMAN NUTRITION

Doctor of Philosophy

THE INFLUENCE OF DIETARY NITROGEN INTAKE ON UREA-NITROGEN SALVAGE IN THE COLON

by Tracey Suzanne Meakins

In the evaluation of nitrogen metabolism, many investigators use urinary urea excretion as a measure of amino acid oxidation. However, this does not take into account the proportion of urea production which passes to the colon where it is hydrolysed into ammonia by the colonic bacteria which contain the urease enzyme. The nitrogen may then either be reincorporated into the body pool as ammonia or as bacterially derived amino acids. This process is termed urea salvage and can be measured quantitatively using the stable isotope $^{15}N^{15}N$ urea.

Enhanced urea salvage is an adaptation to protein restriction. As protein intakes falls, urea salvage increases in order to make up for the shortfall in nitrogen intake. However, there is a critical point at 35g protein/day intake in man below which the salvage system fails and this is recognised as the minimum protein requirement. Hence, above this intake, urea salvage is switched on and contributes to the maintenance of nitrogen balance.

The aim of this thesis was to examine the influence that nitrogen intake has on the control of urea salvage. A series of experiments determined that the primary influence on the rate of urea salvage is the appearance of urea in the body pool which, in turn, is related to the total nitrogen intake in the diet. Nitrogen balance can be improved on a low protein intake by addition of non-specific nitrogen, as urea, to the diet which is incorporated into the body by colonic hydrolysis. With supplemental glycine, nitrogen balance was improved by replacing the deficit in glycine directly without an increase in urea salvage. Urea salvage has also been shown to respond to nitrogen intake on a diurnal basis, increasing during the overnight fast, despite a constant rate of urea production over a 24 hour period.

These experiments were carried out at the boundary of successful adaptation and relatively large amounts of urea were required to improve nitrogen balance, suggesting that there may be other limiting factors for urea salvage. The influence of sulphur amino acids was investigated; however, under these conditions, methionine did not improve nitrogen status and reduced the salvage of a urea supplement. This may have been due to the nitrogen intake level, an inability of the colonic bacteria to utilise the methionine or toxic effects of excess methionine.

Urea salvage is an integral process in nitrogen metabolism, particularly when protein intake is restricted. Recycled nitrogen should be included in models of protein turnover when considering protein recommendations for populations.

The Pobble who has no toes
Had once as many as we;
When they said, 'Some day you may loose them all';-
He replied,- 'Fish fiddle de-dee!'
His Aunt Jobiska made him drink
Lavender water tinged with pink,
For she said, 'The world in general knows
There's nothing so good for a Pobble's toes!'

Edward Lear, 1871

Contents:
page

List of Tables:

List of Figures:

Abbreviations

AA	amino acid
ADP	adenosine diphosphate
AMP	adenosine monophosphate
Au	Urea appearance
BMR	basal metabolic rate
CO_2	carbon dioxide
CV	coefficient of variation
d	day
DIW	deionised water
DRV	dietary reference value
d30	dose of urea-30 ($^{15}N^{15}N$ urea)
EDTA	ethylenediaminetetraacetic acid
Eu	urea excretion
FAO	food and agriculture organisation
g	gram
GDH	glutamate dehydrogenase
h	hour
HCl	hydrochloric acid
HT	height
HYPO	alkaline hypochloride
H_2O	water
I	ingestion
Iu	urea ingestion
IMP	inosinate
IV	intra venous
kg	kilogram
kJ	kilojoule
LBM	lean body mass
LiOH	lithium hydroxide
LiOBr	lithium hypobromite
LP	low protein
LP-M	low protein and methionine
LP-U	low protein and urea
LP-UG	low protein, urea and glycine
LP-U1/U2	low protein and urea
mg	milligram
ml	millilitre
N/N_2	nitrogen
nm	nanometre
NAD/NADH	nicotinamide adenosine dinucleotide
NaOH	sodium hydroxide
Na_2SO_4	sodium sulphate
NEN	non-essential nitrogen
NH_3/NH_4^+	ammonia/ammonium ions
ONL	obligatory nitrogen loss
OOL	obligatory oxidative loss
P	protein
PEG	polyethylene glycol
PNG	Papua New Guinea
PNP	phenol nitroprusside
Pr	urea production from recycled nitrogen
Pu	endogenous urea production
RDA	recommended daily allowance

REE	resting energy expenditure
REF	reference diet
RNI	reference nutrient intake
rpm	revolutions per minute
RQ	respiratory quotient
S	synthetic pathways
SEM	standard error of the mean
sl	slice
T	hydrolysis
$\dot{V}CO_2$	rate of carbon dioxide expired
$\dot{V}O_2$	rate of oxygen used
WT	weight
µg	microgram
µl	microlitre
^{13}C	labelled 13 C carbon (stable isotope)
^{14}N	nitrogen (normal atom)
^{15}N	labelled 15N nitrogen (stable isotope)
^{18}O	labelled 18 0 oxygen (stable isotope)
^{0}C	degrees centigrade

Acknowledgements

This is the place where I get to thank everyone that has contributed to the research in this thesis and the well-being of the writer. Everyone in the department has played their part but particular thanks must go to the following:

The most profound influence was Prof, whose enthusiasm, intellect and sense of humour was inspirational. Thank you for patiently waiting for my brain to catch on. Thanks also go to the 'old hands' Ritchie,Irene, Jane and Simon for their invaluable technical advice and support, and to Sarah for doing the anthropometric measuring. Thank you to Oxford Pharmaceuticals for supplying the methionine and glycine. To my male and female subjects who donated their time and bodies, I'd like to express my sincere gratitude as this thesis would not have been possible without you, even though some of you have had your revenge.

Most thanks must go to the people who kept life so busy and happy: Rafe, Sarah, David, Bob, Kerry, Gary, Jo, Angela, Becky especially. To everyone who took me out and bought me a drink when I needed it; Emma, Catherine, Anthony, Steve and the Monte wardens, thanks. Special thanks must go to my partner in crime, Amanda. We kept each other going through adversity and if that doesn't make us friends for life, nothing will!

Finally, I'd like to acknowledge the support of my family throughout my eternal student life. To Mum and Nan for always welcoming me home with a hug and a hot meal, and to Dad for mending my car. Justin, who gave me a home and best friend. Thank you for your neverending love which was a focus though good and bad. And to Grandad - this is my opportunity to thank you for all the support, interest and encouragement that you've shown from my first calculator to University career. I hope that I've fulfilled all your hopes for me. This thesis is dedicated to you.

Publications to date:

Meakins T.S. & Jackson A.A.
Salvage of exogenous urea-nitrogen enhances nitrogen balance in normal men consuming marginally inadequate protein diets.
Clin Sci (In press)

Meakins T.S. & Jackson A.A. (1994)
Urea appearance:critical determinant of urea-nitrogen salvage on low-protein diets.
Proc Nut Soc **53**, 196A

Meakins T.S. & Jackson A.A. (1995)
Diurnal cycling in urea-nitrogen hydrolysis
Proc Nut Soc **54**, 137A

Meakins T.S. & Jackson A.A.
The influence of sulphur amino acids on urea-nitrogen salvage
Proc Nut Soc (In press)

Persaud C., Meakins T.S. & Jackson A.A.
Nitrogen or protein intake and urinary 5-oxoproline excretion in adults
Proc Nut Soc (In press)

Meakins T.S. & Jackson A.A.
The influence of glycine on urea-nitrogen salvage
Proc Nut Soc (In press)

CHAPTER 1

INTRODUCTION

Our bodies are composed of the elements which surround us in the planetary ecosystem. In order to replace substances lost from metabolism, our diet contains the basic compounds for life. Nitrogen is especially crucial as it can be found in the functional molecules in the body. Protein is the major dietary source for nitrogen and sulphur, but nitrogen can also be derived from other sources such as nucleic acids and vitamins.

In order for a healthy functional state to be maintained, substrate supply must balance metabolic demands. The nature of the demand for nitrogen is governed at different levels by genetic disposition, physical activity, pathology and dietary supply. Physiological requirement, and hence the functional demand, is necessary for maintenance of the protein synthesis/oxidation cycle, to balance obligatory nitrogen losses and to account for any net deposition in tissue during growth, pregnancy or lactation. There are also intrinsic requirements for amino acids in non-protein pathways and for metabolic regulation.

It is important to distinguish between the concepts of protein, amino acids and nitrogen when discussing requirements. In simple terms, protein, amino acids and nitrogen are different supplies of the substrate ultimately required to meet metabolic demand. Proteins comprise of combinations of the twenty amino acids important in nutrition, some of which are, under certain metabolic states, unable to be synthesized in the body and hence, must be supplied in the diet. Estimations of protein requirements are mostly considered in terms of the essential amino acids. However, it may be more prudent to think in terms of total nitrogen requirements. Nitrogen intake consists of two components; the essential amino acids and non-essential nitrogen. Non-essential nitrogen is defined as additional sources of nitrogen to that from the essential amino

acids and includes non-essential amino acids, excess essential amino acids after requirements have been met and other non-proteinous sources such as urea or ammonium salts.

Substrate supply does not simply come from dietary intake. In the case of nitrogen there are three potential sites of supply. The majority of nitrogen comes from the degradation of body or dietary protein and amino acids. However, a further input comes from the urea-nitrogen salvaging system. It has been recognised since the 1950's that urea produced from amino acid degradation is not all excreted via the urine. A proportion passes to the colon where it is hydrolysed to ammonia by the bacterial microflora which contain the urease enzyme. This nitrogen source is incorporated into the body's nitrogen pool as amino acids or reformed into urea in the urea cycle. The system can be measured quantitatively using stable isotopic labelling and this can give an insight into the mechanisms behind nitrogen handling in the body.

The salvage of nitrogen by the colonic microflora is an adaptive mechanism in the face of dietary nitrogen restriction, increasing in order to compensate for a deficiency of nitrogen intake. Hence, the urea salvage may reflect the essentiality of different nitrogen sources in the diet. The aim of this thesis is to investigate the interaction of non-essential nitrogen sources in the diet with the urea-nitrogen salvaging system and hence, add some understanding to the dilemmas still surrounding the determination of nitrogen requirements in humans.

CHAPTER 2

LITERATURE REVIEW

Nitrogen metabolism is a fundamental aspect of one of the body's main aims - to maintain the internal milieu. The ultimate product of nitrogen metabolism, ammonia, is toxic and hence has to be removed if the system is to function efficiently. As the balance between the body's demand for nitrogen can be met over a range of differing dietary intakes, the absolute minimal requirement remains unclear.

2.1 Historical Developments in Nitrogen Metabolism

As protein is the most abundant nitrogenous compound, the history of nitrogen metabolism is bound to the distribution of proteins in nature and ultimately to the discovery of amino acids.

Out of the scientific revolution in the second half of the eighteenth century came the classification of nitrogen as an independent element by Rutherford in 1772 (Munro 1964). It was given the name 'nitrogen' by Chaptal in 1790 in a french text on chemistry. In the same year Lavoisier carried out the first metabolic experiment on nitrogen from which he concluded that gaseous nitrogen plays no part in nitrogen metabolism.

Dietary studies were first carried out by Francois Magendie in 1816. He concluded that nitrogenous constituents of the body are derived from food and thus dietary nitrogen is essential for survival; his enquiring mind also led him to state fundamental views on the dynamic state of body tissues. The next advance in nitrogen metabolism came from Justus von Liebig who, taking a concept developed by Gerald Maulder in 1839, explored the field of protein chemistry and ascribed a general formula to protein structure. He had no concept, however, of the possibility of digestion and reconstruction of dietary protein.

From the school of biochemical studies set up by Liebig in 1852 came Carl Voit who took the idea of nitrogen intake and output studies published by Boussingault in 1844 and developed

the concept of nitrogen balance as a method of studying the body's nitrogen status. Along with Bischoff, he determined that in healthy subjects input and output were equal, giving a state of nitrogen equilibrium. Following the refutation of Liebigs hypothesis that protein was the source of muscle energy, Voit developed the concept of a labile pool of body protein.

The recognition of amino acids as structural components of proteins opened the way for new concepts of the nutritional value of proteins. Although Branncot had observed amino acids to be products of acid hydrolysis of proteins in 1820, it was not until Kossel and Kutcher developed the analysis of individual amino acids in 1900 that the study of protein quality could begin. The establishment of a link between defects in nutritional value and amino acid composition of proteins was determined in 1906 by Willcock and Hopkins, to be confirmed by Osborne and Mendel in 1914. Both groups added tryptophan and lysine to a corn protein which made it capable of sustaining growth. This had limitations which existed until the classical feeding experiments of Rose in 1938 which led to the classification of amino acids as essential or non-essential. In 1909 Karl Thomas established a procedure for measuring biological values of proteins by nitrogen balance determination which ultimately resulted in the indication of quality known as 'chemical score' by Mitchell and Block in 1946.

The present chapter in the history of nitrogen metabolism began with the discovery of isotopes. This allowed Schoenheimer to disprove the theories of Folin who postulated that protein metabolism was separated into endogenous and exogenous components. He extensively used stable isotopes to solve metabolic problems which culminated with the technique of using the isotope ^{15}N, usually incorporated into an amino acid, to give an insight into nitrogen metabolism in the body.

2.2 Demand for Nitrogen in the Body

When considering the body's requirements for nitrogen, it must be understood that elemental nitrogen cannot be handled by the body's metabolism which deals with nitrogen containing compounds. Although a wide range of these are fundamental components of the body, the nitrogen pool is quantitatively dominated by body proteins. Furthermore, virtually all of the body's nitrogenous compounds can be derived from amino acids. Hence the body's nitrogen demand is centralised around protein function.

Proteins are amongst the most important compounds in metabolism and without them life would be impossible. The name itself underlines this, being derived from the greek word *proteios*, meaning 'of the first rank'. They play essential roles controlling metabolism as enzymes and hormones, maintaining fluid and acid-base balance, protecting the body as antibodies and clotting agents, playing important roles in transport, structure and mobility, and even contribute to senses as visual pigments. With an appreciation of the diversity and importance of proteins, it brings into significance the essentiality of protein as a dietary component.

Other nitrogenous compounds are equally vital to existence. Nucleic acids, made up of purines and pyrimidines, contain the information for our composition and function and there are numerous small nitrogenous compounds which regulate metabolism, for example, adenosine triphospate, choline and porphyrins. Hence, the body's demand for nitrogen is of primary importance.

Demand for nitrogen is dominated by the demand for protein. However, the body's protein is in a dynamic state, continually being synthesized and degraded - a process known as protein turnover, necessary because of the small amounts of free amino acids available. Hence body protein pools are mobilised as different amino acids are required for a diversity of proteins. The process of protein turnover is sensitive to influences such

as dietary intake and activity (Waterlow & Jackson 1981, Waterlow 1984). The overall effect is the turnover of each individual amino acid in proportion to its occurrence in protein. Ultimately this leads to the loss of amino acids freed from one protein but not required for another and is termed the 'obligatory oxidative loss' (OOL)(Millward & Rivers 1988).

This is one component of the 'obligatory nitrogen loss' (ONL), defined as the amount of nitrogen lost from the body (in urine, faeces, sweat, etc) when foods with essentially no protein (but otherwise adequate) are consumed. This is equivalent to about 0.55mgN/kJ basal metabolic rate in adults (FAO 1985) and is remarkably consistent in the literature (Bodwell *et al* 1979, Uauy *et al* 1978, Scrimshaw *et al* 1972, Young & Scrimshaw 1968). The ratio of obligatory nitrogen losses to BMR is significantly lower in infants and young children (Fomon *et al* 1965, Huang *et al* 1980). The obligatory nitrogen loss forms the basis of the factorial method for estimating nitrogen requirements; ie. the requirement was considered to be the amount needed to replace individually variable endogenous losses, after adjustments for the inefficiency of dietary protein utilisation and the biological quality of the dietary supply.

Minimum nitrogen intakes required to maintain the bodies nitrogen equilibrium are greater than the obligatory nitrogen loss. Other demands for nitrogen account for net deposition of tissue in growth, pregnancy and lactation, nitrogen required for other non-protein pathways (such as nucleic acid metabolism) and for what is termed the 'anabolic drive' (Millward & Rivers 1988). This is the regulatory influence that amino acids have on the organism. The magnitude of this is unknown but it can be seen to be involved in the hormonal control of growth and immunocompetence.

The nature of the demand is most closely influenced by the rate of protein synthesis which, in turn, is established by a combination of genetic regulation and state of maturation. There are also several variable elements controlling an individual's demand such as dietary intake, the general

environment and the habitual level of activity (often summarised as 'lifestyle'). With dietary intake, satisfaction of metabolic demand for protein is greatly influenced by energy intake and the interactions of the two have been studied at length (Scrimshaw & Schurch 1991). However, intake of food in general has to be handled and this also contributes to the metabolic demand.

One approach to estimating the requirements for nitrogen is the 'nitrogen balance technique'. Nitrogen balance studies have proved useful and provided important data regarding requirements but this widely used technique does appear to have limitations (Hegsted 1976, Young 1986). Estimation of balance involves the determination of the difference between the intake of nitrogen and that lost from the body via urine and faeces. Allowances are made for losses by other routes on the basis of a limited number of published studies (FAO 1985); however, total nitrogen losses are extremely difficult to assess and there are compounding effects on nitrogen balance due to the influence of dietary energy. Experimentally, dietary periods are often short term and it is relatively unclear how long is required for the body to reach an equilibrium in nitrogen excretion when intake is altered. Nitrogen equilibrium can be achieved over a range of protein intakes, however, at higher protein intakes apparent nitrogen balance appears to reach unrealistic positive balances. It is also questioned that dietary nitrogen is completely available for metabolism, and studies claim that the efficiency of utilisation maybe only somewhere in the order of 70% (Calloway & Margen 1971, Young *et al* 1971). Hence, nitrogen balance data does not necessarily provide an adequate measure of the status of body nitrogen nor does it offer a sole means for establishing requirements. In view of these limitations in the conduct and interpretation of this technique, it is important that alternative methods are applied. The answer undoubtedly lies in the use of stable isotopes.

Whatever the method used to estimate requirements, the demand

for nitrogen is undeniably linked to the loss of nitrogen from the body.

2.3 Elimination of Nitrogen from the Body

In order to maintain optimal function the composition of the body fluids must be kept within a restricted range. The kidneys are the chief organs of the body operating to maintain constant homeostasis of the internal environment through the excretion of a water solution, the composition of which helps to correct tendencies toward abnormal changes in the body fluids. Hence, the kidneys excrete substances present in excessive quantities, waste products and toxic chemicals. In comparison, they can also act to conserve valuable materials such as glucose. The study of urine therefore contributes greatly to our understanding of metabolism (Allison & Bird 1964).

In the case of nitrogen metabolism, this is particularly relevant as the majority of the end products of nitrogen metabolism are lost in the urine, although some is lost through the faeces or dermally. The major nitrogenous excretion product is urea which is lost totally in the urine and accounts for 87.5% of nitrogen output.

In addition to the analysis of organic materials for nitrogen, the nineteenth century also saw the isolation of several compounds of considerable interest in the study of nitrogen metabolism (Munro 1964). In 1773 Rouelle identified urea in the urine; in 1823 Prevost and Dumas showed that urea accumulated in the blood when the kidneys of rabbits or cats were removed. This demonstrated that urea was not synthesised in the kidneys, and the liver was suggested as the site of formation. Liebig later contributed by drawing attention to urea as an end product of protein metabolism. He further developed his misconceived view that muscle protein was the fuel for exercise by concluding that the nitrogenous components of the diet were first converted into tissue before being

broken down to form urea. Voit took up this point and tried to improve methods of measuring nitrogen output in the urine. In 1860 he published results of feeding experiments where he found that, with increased feeding, less nitrogen was lost from the body. He also published data that showed exercise was not associated with a significant increase in urea output. In 1882 Schroeder showed that urea was formed on perfusing the liver with ammonium salts; however, the exclusive synthesis of urea in the liver remained inconclusive for some time. When a procedure for total hepatecomy was developed, Bollman demonstrated in 1924 that removal of the liver results in a complete suppression of urea synthesis. This was followed in 1932 by the revolutionary studies of Krebs and Henseleit which led to the formulation of the arginine-ornithine cycle as a mechanism of urea synthesis. This was the first cyclic metabolic pathway to be described. The problem of the mechanism by which the amino groups were obtained was solved in 1937 when Braunstein and Kritzman discovered transaminases.

Amino acids in excess of those needed for protein synthesis cannot be stored, nor are they excreted; rather, the α-amino group is removed and the resulting carbon skeletons are converted into major metabolic intermediates, such as acetyl coA or pyruvate. The α-amino groups are converted to ammonium ions by oxidative deaminations of glutamate either directly or after transmination to α-ketoglutarate. Serine and threonine can be converted directly into ammonium ions by deamination by dehydratases because these amino acids contain a hydroxyl group attached to a ß-carbon.

An alternative source of ammonium ions may be from deamination of AMP to IMP in the purine nucleotide cycle (McGiven & Chappell 1975). Hepatic ammonia is also regulated by a third pathway. Glutamate has two fates, either the formation of aspartate by transamination with oxaloacetate or production of ammonia by deamination. It has been concluded that the activity of glutamate dehydrogenase in the liver may not necessarily be a primary source of ammonia (Jahoor *et al*

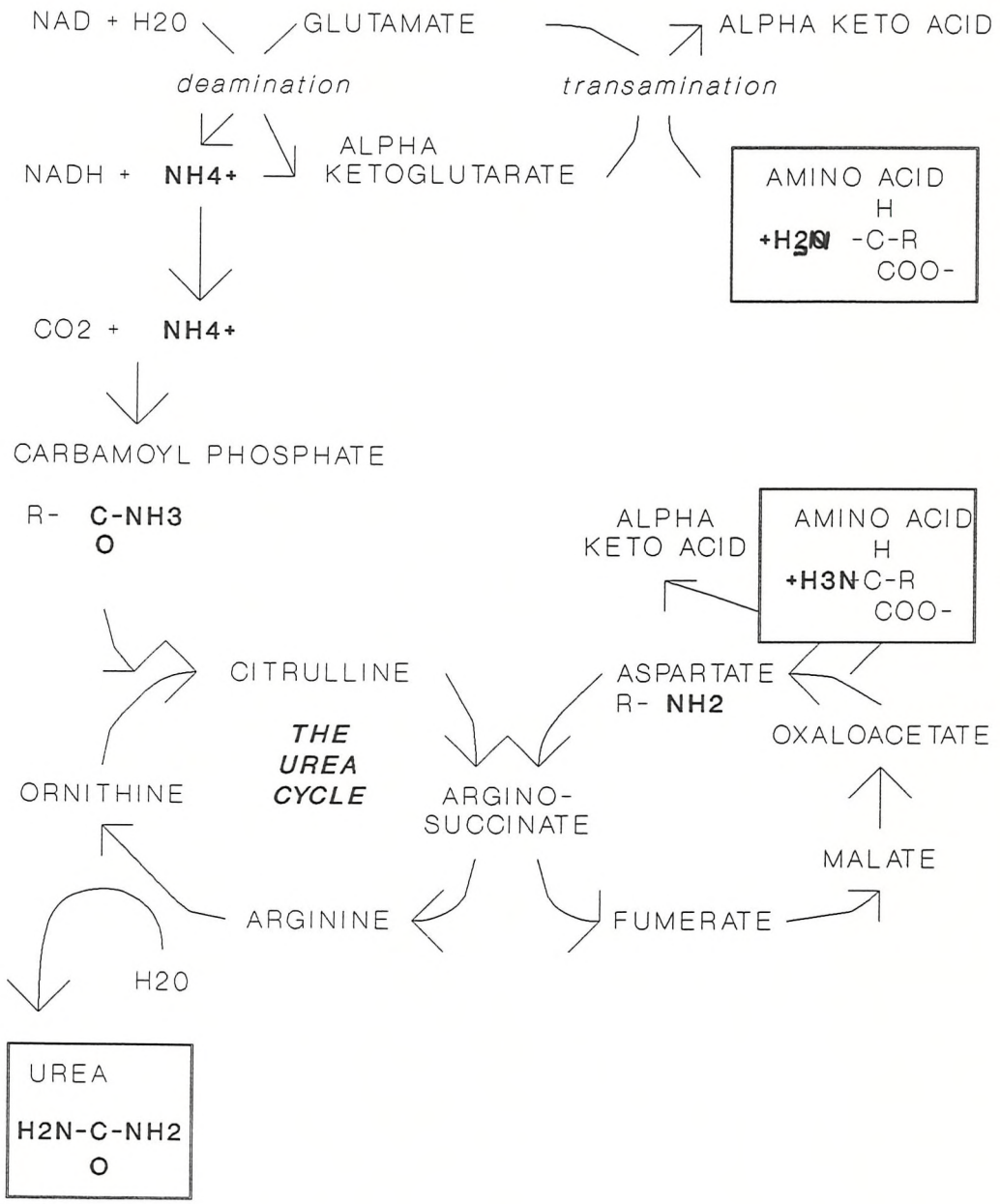
1988). Circulating ammonium ions and glutamine may be more important sources of hepatic ammonia.

Ammonia is produced in significant amounts in the liver and kidney and is readily diffusible; however, circulating levels must be kept low due to its toxic effects. Hyperammonaemia is relatively rare, occurring only in patients with liver disease (Hsia 1974) or due to errors of urea biosynthesis (Jackson, M *et al* 1986, Walter & Leonard 1987). Toxic effects are manifested in altered cerebral metabolism as in hepatic encephalopathy (Phillips *et al* 1952) and dysfunctions in regulatory enzymes such as phosphofructokinase and pyruvate kinase (Passonneau & Lowry 1964, Pogson 1968).

Some of the ammonia formed is consumed in the biosynthesis of nitrogenous compounds; however, in most terrestrial vertebrates, the excess ammonia is converted into urea via the urea cycle (Figure 2.1) and then excreted.

One of the nitrogen atoms of the urea synthesised by this pathway is transferred from an amino acid, aspartate. The other nitrogen atom and carbon atom are derived from the ammonium ion and carbon dioxide. Ornithine is the carrier of the carbon and nitrogen atoms and the intermediate precursor of urea is arginine. This process is also a device by which the liver can contribute to the maintenance of pH homeostasis by balancing the conversion of ammonium ion and bicarbonate ions into urea (Atkinson & Bourke 1984, Haussinger 1990).

Figure 2.1 The Urea Synthesis Pathway.



Hence, the majority of nitrogen lost from the body is as urea, formed directly from ammonium ions produced by amino acid deamination and transmination. The second largest body component consisting of nitrogen, the nucleic acids, also contributes ammonia for the urea cycle. Some free ammonia is also lost in the urine. In the degradation of purines, uric acid is formed in an oxidative reaction; this is another source of nitrogen loss in the kidney. In terrestrial reptiles and birds, this is the major nitrogenous excretion product, as it requires less water loss than the highly soluble urea (Bender 1985). Creatinine is also excreted in the urine in relatively constant amounts. It arises non-enzymatically by the cyclization of creatine and creatine phosphate in muscle, and the amount formed depends largely on muscle mass. Other nitrogenous losses in the urine include: small amounts of creatine in children, post-menstrual women and in muscle wasting disorders; trace amounts of low molecular weight proteins and amino acids; small quantities of the catabolic products of vitamins.

In addition to nitrogen loss via the urine, a smaller amount is loss in faeces and through dermal losses. The nitrogen lost in the stool comes from four primary sources: unabsorbed food nitrogen, cells of the mucosal lining of the intestine, unabsorbed gastrointestinal secretions and the bacterial microflora. This source of nitrogen loss can account for between 8-23mgN/kg daily (Calloway *et al* 1971, Bodwell *et al* 1979, Uauy *et al* 1978). Dermal losses are less easy to measure but include losses of nitrogen in sweat, hair and nails, and other secretions, such as menstruation, semen and saliva. In most cases these losses are taken to be 8mgN/kg daily for adults (FAO 1985).

In conclusion, the body has a fundamental need for nitrogen to replace that lost from the numerous nitrogenous metabolic pathways. The nitrogen status of an individual is determined by the extent of the demand; but the question arises as to how

the body receives a supply of nitrogen?

2.4 Supply of Nitrogen to the Body

In order to fulfil the body's demand for nitrogen, a constant supply of nitrogen is needed to maintain the nitrogen balance of the individual. There are three potential sources of nitrogen available: dietary intake, nitrogen released from nitrogenous compounds during metabolism (ie. amino acids released from protein turnover), and a third source from colonic bacterial metabolism.

Dietary nitrogen consists primarily of proteins and their constituent amino acids. Plants can synthesise all the amino acids they need from simple inorganic compounds and, in the case of legumes, from elemental nitrogen. However, animals are unable to do this because they cannot synthesise the amino (NH_2) group. So in order to obtain the amino acids necessary for building protein, they must eat plants or other animals which, in their turn, have lived on plants. The proteins ingested from these sources go through a process of digestion and absorption before they are in a form useful to metabolism, generally as amino acids.

The hydrolysis of proteins begins in the stomach. The cells lining the stomach secrete a gastric protease in its inactive form (pepsinogen). The hydrochloric acid of the stomach activates pepsinogen, creating pepsin, which can cleave large polypeptides. When these small polypeptides enter the small intestine, pancreatic and intestinal proteases trypsin and chymotrypsin, and carboxypolypeptidase hydrolyse them into short oligopeptides, tripeptides, dipeptides and amino acids. Then peptidases on the surface of the epithelial cells hydrolyse the tripeptides and dipeptides into amino acids - the final products of protein digestion. Amino acids are transferred to pumps, which carry them to the interior of the intestinal cells for passage into the blood.

The role of protein in food is not to provide body protein directly but to supply the amino acids from which the body can make its own proteins; however the human body has limited powers of conversion of one amino acid to another. This is achieved in the liver, by the process of transamination, whereby an amino group is shifted from one molecule to another under the influence of amino transferases, the coenzyme of which is pyridoxal phosphate (a derivative of vitamin B6). Under different conditions some amino acids cannot be made by the body itself and therefore must be obtained from the diet. These amino acids are known as the essential amino acids.

2.5 The Essentiality of Amino Acids.

During the early part of the century, the individual amino acids were identified and isolated. This enabled preparation of diets in which protein was replaced by a mixture of amino acids that could support growth in rats and maintain human subjects (Rose 1938, 1957). Then by deleting one amino acid after another from these diets, Rose was able to distinguish between amino acids that could and those that could not be synthesised by mammals. This gave rise to the classification of amino acids into two groups, the essential and non-essential amino acids. This classification became widely accepted but has since been revised.

Further research began to show that some amino acids did not always fit one or other category. For example, histidine is necessary to maintain the pool of carnosine in muscle and, when removed from the diet, deficiency symptoms such as reduced plasma albumin, increased serum iron and skin lesions became apparent in normal and uraemic men (Kopple & Swendseid 1975). Also, arginine is not synthesised in sufficient amounts by the young of most species (Visek 1984). So the differentiation between essential and non-essential amino acids is not clear cut and in 1983 Jackson proposed a more flexible approach to take into account differing requirements in pathology and with

changing intakes (Jackson 1983). He suggested an alternative system of classification in which the assessment of the essentiality of an amino acid is based on dietary availability of the carbon skeleton and the body's ability to synthesize this, along with the body's capacity to aminate to available carbon skeleton. Hence a classification of four types of amino acid was derived.

In the same year, Harper made the point that non-essential amino acids need to be obtained in the diet under many conditions and hence should be termed dispensable amino acids as this term does not carry the implication that these amino acids are not required in the diet (Harper 1983). During protein turnover amino acids interrelate in groups independent of their essentiality and this may be seen as a nutritional rather than metabolic concept.

In 1987 Laidlaw and Kopple again questioned the concept of indispensable amino acids as development of enteral and parental nutrition prompted reexamination of amino acid requirements, especially during periods of infection or injury. In periods of disease, further amino acids become indispensable and the effect of non-essential nitrogen is also considerable. In conclusion, a modification of Jackson's classification is offered in which a fifth group of acquired indispensable amino acids is added to include amino acids that become indispensable in states of metabolic disorder or stress (Table 2.1). These authors show the need to appreciate that a more open approach is required to amino acid classification as requirements alter with differing physiological states.

Table 2.1 - Classification of the Amino Acids

CLASSIFICATION	DESCRIPTION	EXAMPLES
1. Totally Indispensable	Adverse nutritional or metabolic effects if deleted from the diet -no metabolic precursor can be substituted	lysine threonine
2. Carbon Skeleton Indispensable	Indispensable except that the ketoacid or hydroxyacid can be substituted. Deficiency manifested by rapid development of negative nitrogen balance	histidine isoleucine leucine methionine phenylalanine tryptophan valine
3. Conditionally Indispensable	May become indispensable in the absence of the dietary intake of its precursor	tyrosine cysteine ornithine citrulline
4. Acquired Indispensable	Can become indispensable in states of metabolic disorders or severe stress	
	- immature synthetic processes in preterm infants	cysteine tyrosine taurine
	- genetic disorders of enzyme function	arginine citrulline cysteine tyrosine
	- acquired disease states such as liver failure, Reyes syndrome	arginine cysteine citrulline tyrosine
	- large amino acid intake I.V. nutrition	arginine
5. Dispensable	Carbon skeleton readily synthesized and readily transaminated	alanine glutamate aspartate

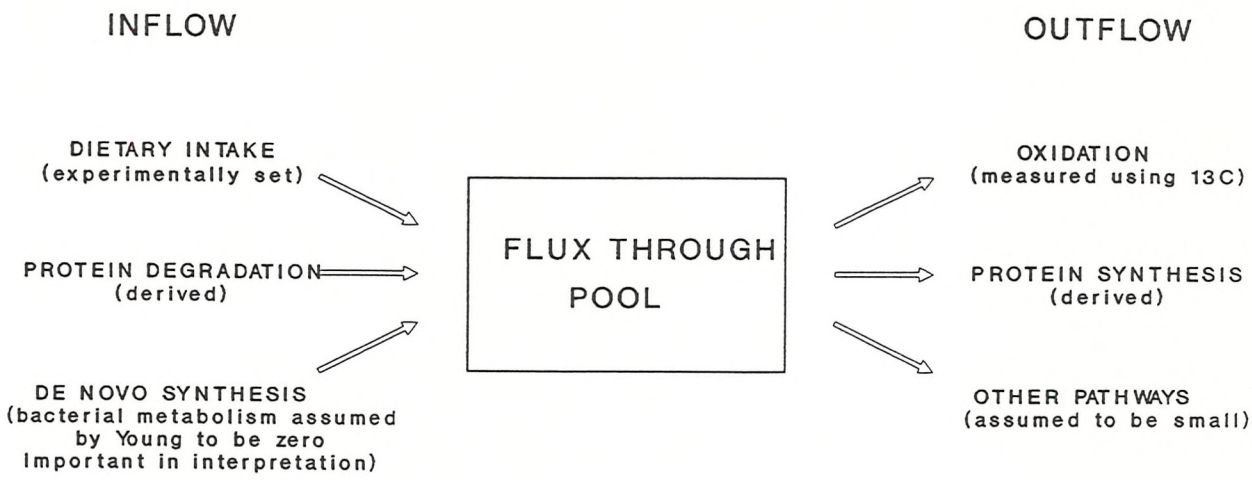
It has also been proposed that the ability to synthesize the dispensable amino acids is, like all biological functions, genetically determined (Bessman 1979). The Justification Theory states that the individual creates their own phenotypic distribution of amino acids from the diet.

Recent observations add another dimension to the question of amino acid essentiality. Colonic microflora are able to fix ammonia as bacterial amino acids which are then available for absorption. The extent to which the human can utilise this source of nitrogen is currently under investigation but there is considerable evidence to suggest that this is an intrinsic part of metabolism. The use of tracer isotope studies has shown that after administration of labelled urea, ^{15}N enrichment is found in plasma amino acids (Rose 1955, Giordano 1968). Surprisingly, small amounts of label were found in the essential amino acids, a result confirmed by the findings of Tanaka *et al* in 1980. She showed this to be due to the action of the microflora, as antibiotics abolished the appearance of label in all amino acids. Sheng gave subjects ^{15}N ammonium chloride and reported the label in the imidazole ring of the histidine molecule obtained from plasma proteins; the high enrichment of ^{15}N from faecal bacterial cells suggested a possible bacterial site of histidine synthesis (Sheng *et al* 1977). More recently, Torrallardona has demonstrated the movement of ^{15}N into lysine in the pig and rat when the sole source of nitrogen was ammonia (Torrallardona *et al* 1993a, 1993b). This apparent incorporation of bacterial lysine into the hosts amino acid pool has now been established in malnourished children in functionally significant amounts (Yeboah *et al* 1995). The question now is philosophical - are the bacteria an integral part of the host metabolism and hence the host is synthesising essential amino acids, making them dispensable; or are the bacteria external to the host as they are inside the gastrointestinal cavity, merely a tunnel through the host, and hence not internal. Whichever, this process is another potential source of amino acids, and nitrogen to that of dietary intake and should not be ignored in questions of requirement estimations.

The requirements for essential amino acids have been assessed by measuring nitrogen balance in adults, starting with the classical work of Rose (Rose 1957) and also by determining the

amounts needed for normal growth and nitrogen balance in infants and children, by comparison with observed intakes of good quality proteins (Holt & Snyderman 1965, FAO 1985). A different approach involves the use of isotope labelled amino acids. The theory is that an amino acid for which there is limited availability will be conserved, with a decrease in its catabolism; thus the rate of amino acid input which leads to an increase in its oxidation is just above the individuals minimum requirement for an amino acid. Young has suggested that the figures currently used for essential amino acid requirements based on Roses experiments may be too low when compared to values he has derived by this technique (Young & Bier 1987, Young *et al* 1989, Young & Pellet 1990, Marchini 1993). In answer to this theory, an alternative model was proposed that not only accounts for oxidative losses, but also makes provision for demands for maintenance of nitrogen status and for the regulatory influence termed the 'anabolic drive' (Millward & Rivers 1988, Millward *et al* 1989). The model also recognises the influence of diurnal cycling and non-essential nitrogen towards essential amino acid requirements. Young's model has been criticised as there is no allowance for bacterial contributions to nitrogen metabolism by colonic recycling (Jackson 1993a, Jackson 1995) and a further simple model has been proposed (Figure 2.2).From this is can be seen that, when values for de novo synthesis are ignored and outflow to other pathways considered too small to be significant, these can influence the determination of requirements from amino acid oxidation.

Figure 2.2 Model for the assessment of amino acid kinetics



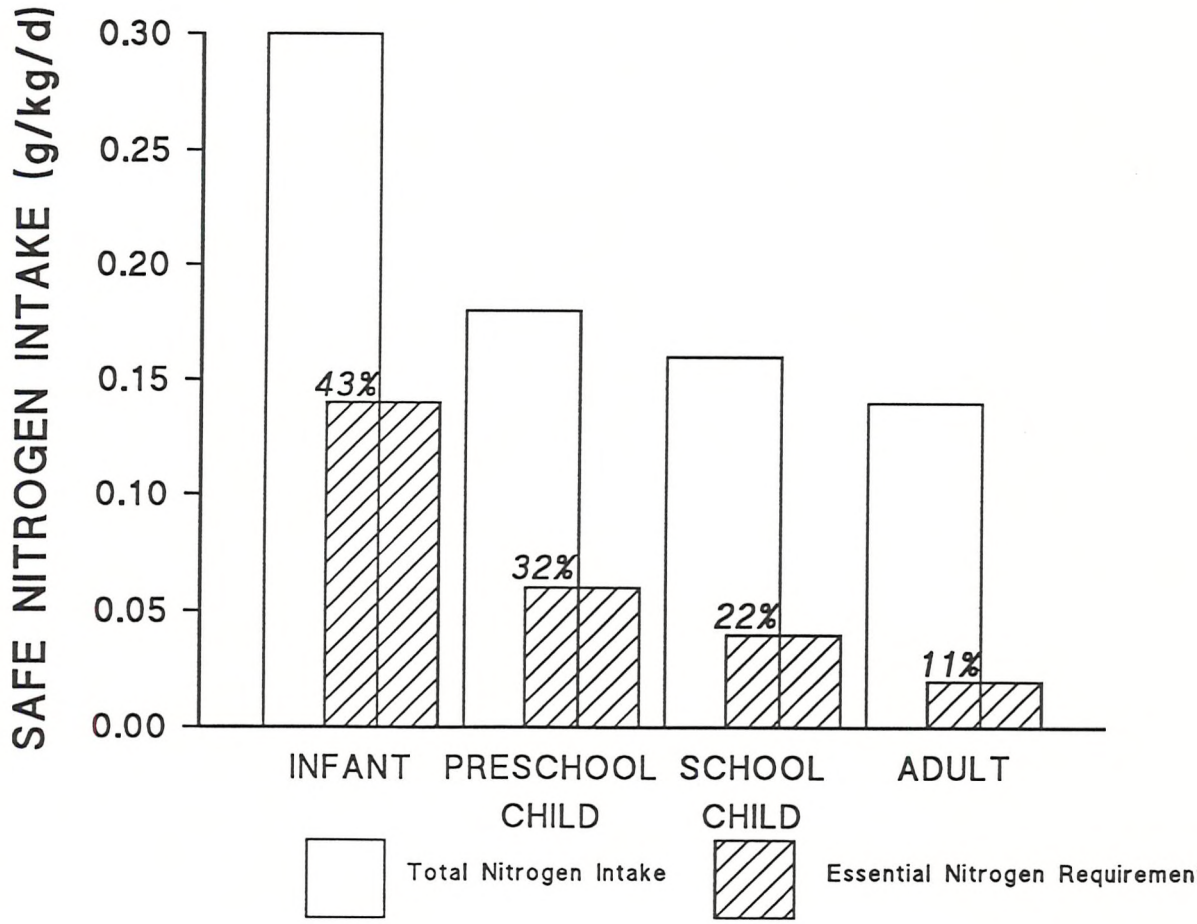
Another factor which influences the essentiality of amino acids, and their requirements is the amount of total nitrogen present ie. the ratio of essential to non-essential nitrogen.

2.6 Non-Essential Nitrogen

The physiological demand for nitrogen can be met by the supply of two sources of nitrogen - essential nitrogen (or the indispensable amino acids) and non-essential nitrogen. Non-essential nitrogen can be defined as additional sources of nitrogen to that supplied by the essential amino acids. This includes excess essential amino acids after requirement has been met, non-essential or dispensable amino acids and other non-protein nitrogen compounds such as urea or ammonium salts.

The importance of non-essential nitrogen is underlined when the proportion in total nitrogen intake is examined (Figure 2.3). It can be seen that the proportion of essential nitrogen in the total is low even in childhood but in adults contributes only 11% of the total requirement. Thus in adults 89% of the requirement for nitrogen is met by non-essential nitrogen (FAO 1985).

Figure 2.3 Requirements for Essential Nitrogen as a proportion of total nitrogen intake in different age groups.



It is generally assumed that natural food proteins provide sufficient nitrogen for the synthesis of non-essential amino acids and requirements are based on the belief that, if essential amino acids are present in suitable quantities, then this will guarantee an adequate nitrogen intake. Numerous studies have, however, demonstrated that in some cases non-essential nitrogen is the limiting factor.

Early studies in this field (Rose 1949) demonstrated that growth is reduced in rats fed only essential amino acids at minimum requirement levels and that supplements of ammonium salts, urea and other nitrogen sources showed a striking acceleration of weight gain. This data shows that with a minimum essential amino acid intake, the limiting factor is the inability of cells to synthesise non-essential amino acids due to a shortage of nitrogen; this is relieved by supplementing alternative nitrogenous compounds. In a further study, Rose recognised the importance of non-essential nitrogen in the human diet, determining that 3.5g of nitrogen is required daily for the synthesis of non-essential amino acids (Rose & Wixom 1955). In the same year he looked more specifically at urea as a nitrogen source and found that growing rats incorporated ^{15}N from labelled urea into several non-essential amino acids, enrichment increasing when protein intakes were lower (Rose & Dekker 1955).

Further to this, Snyderman questioned the theory built up by Mendel that essential amino acids are the limiting factor in protein quality (Snyderman *et al* 1962). Four infants were fed decreasing quantities of milk protein with constant calories in order to decide which was the most limiting amino acid of the diet. It was found that when weight gain and nitrogen retention was eventually altered, this could be restored to normal by supplementing non-essential nitrogen in the form of urea or glycine. Thus non-essential nitrogen appeared to be the limiting factor. Incorporation of this nitrogen was demonstrated by the enrichment of ^{15}N in haemoglobin and plasma protein from ^{15}N urea and ^{15}N ammonium chloride.

Studies then looked extensively at the effects of non-essential nitrogen on nitrogen balance in human subjects in an attempt to determine the minimum proportion of essential amino acids to total dietary nitrogen. Reference proteins were used (such as egg) which have an ideal balance of essential amino acids and the metabolic responses to the isonitrogenous replacement of protein with a non-essential nitrogen source were examined at low levels of protein intake (Huang *et al* 1966, Scrimshaw *et al* 1966, Weller *et al* 1971). It was established that the addition of non-essential nitrogen to diets low in total nitrogen, but with sufficient essential amino acids, improved nitrogen balance (Giordano 1963, Clarke *et al* 1963b, Tripathy *et al* 1970, Garza *et al* 1978).

The greatest contribution to this area of research was given by Constance Kies, who published an interesting series of papers in the 1960' and 1970's. Starting with the idea that the most important determinant of the nutritional quality of a protein is its amino acid composition as compared to the amino acid requirements of the organism consuming it, she investigated the protein quality of cereal proteins. Along with her colleagues, she was able to determine that non-essential nitrogen was the limiting factor in corn protein (Kies *et al* 1963, 1965b, 1967) and conducted a series of supplementary experiments demonstrating the beneficial effects of non-essential nitrogen (Kies *et al* 1967, 1972, Chen *et al* 1967, Korsland *et al* 1977).

Once this phenomenon was established, Kies drew together results from comparative studies looking at the specific source of non-essential nitrogen (Swendseid *et al* 1960, Clarke *et al* 1963, Anderson *et al* 1969, Kies 1974). Using these studies it was possible to derive a rank order of the efficiency of different sources of non-essential nitrogen (Table 2.2). Recently this has been characterised as a system of exchanges (Jackson 1995): if there is a metabolic demand for a defined pattern of amino acids, then the closer the pattern of amino acids in the diet to the demand, the less the total amount of

amino acids required for balance; hence, the greater the amount of nitrogen exchange required, the higher the level of total nitrogen intake needed to maintain balance.

Table 2.2 Order of efficiency of non-essential nitrogen in improving nitrogen balance on low protein intakes

Rank Order for Non-Essential Nitrogen
Non-Essential Amino Acids
Glycine/ Glutamate
Glycine/ Diammonium Citrate
Glycine/ Urea
Diammonium Citrate
Urea

The level of non-essential nitrogen supplementation may effect the requirements for essential amino acids in that they contribute to the total amount of nitrogen. If total nitrogen level is low then essential amino acids not only have to fulfil requirements for essential nitrogen but also for the synthesis of non-essential amino acids and hence, the requirements for essential amino acids would be increased. However, essential amino acids are always required at a minimal level and it is important to stress that non-essential nitrogen does not lower this minimum requirement (Hiramatsu *et al* 1994).

Kies considered the practical implications of the use of supplemental non-essential nitrogen in humans. Some of the most important work related to non-essential nitrogen needs in humans is that concerned with the dietary treatment of uraemic patients (Giordano *et al* 1963,1968, Giovannetti & Maggoire 1964). It indicated that high levels of blood urea nitrogen in uraemia can be reduced, probably through utilisation for amino acid synthesis if small amounts of idealized patterns of essential amino acids are included in protein-free diets or in intravenous feeds. It appears that non-essential nitrogen is important in the nutrition of preterm or low birth weight

infants (Darling *et al* 1993). Current formulae designed for feeding premature infants contain a higher proportion of whey proteins than casein proteins. Whey protein sources have a high non-essential nitrogen content, predominantly as urea, similar to that of human breast milk, whereas cows milk only contains 3-5% of total nitrogen as non-protein nitrogen. The implications for non-essential nitrogen in the nutrition of human being are also of concern in meeting the protein needs of an expanding world population. Requirements stress protein quality in terms of essential amino acid patterns and without taking into account total nitrogen intake these may be incorrect. Potential hazards prohibit the use of urea supplementation in food products and clinical diets (Visek 1972, 1974); however, urea supplementation does offer a useful research method for better understanding of the nutritive role of non-essential nitrogen.

Hence, the essential nature of non-essential nitrogen is an important consideration for the supply of dietary nitrogen. Underlying this is an alternative source of nitrogen for metabolism. When the non-essential source of nitrogen is urea, this can contribute nitrogen to the body by way of the metabolic activity of the colonic microflora which contain ureases. Even endogenous urea produced in the urea cycle is recycled in the colon to provide a further nitrogen source.

2.7 Urea Nitrogen Recycling in the Colon.

Urea was long thought to be the inert metabolic end product of nitrogen metabolism. A turning point occurred in the 1950's when Walser published results from a series of stable isotope studies which suggested that in fact urea is hydrolysed in the gut (Walser & Bodenlos 1959). It was also found that the ^{15}N enriched ammonia produced was reincorporated into newly formed urea. This breakdown was presumed to be due to ureases present

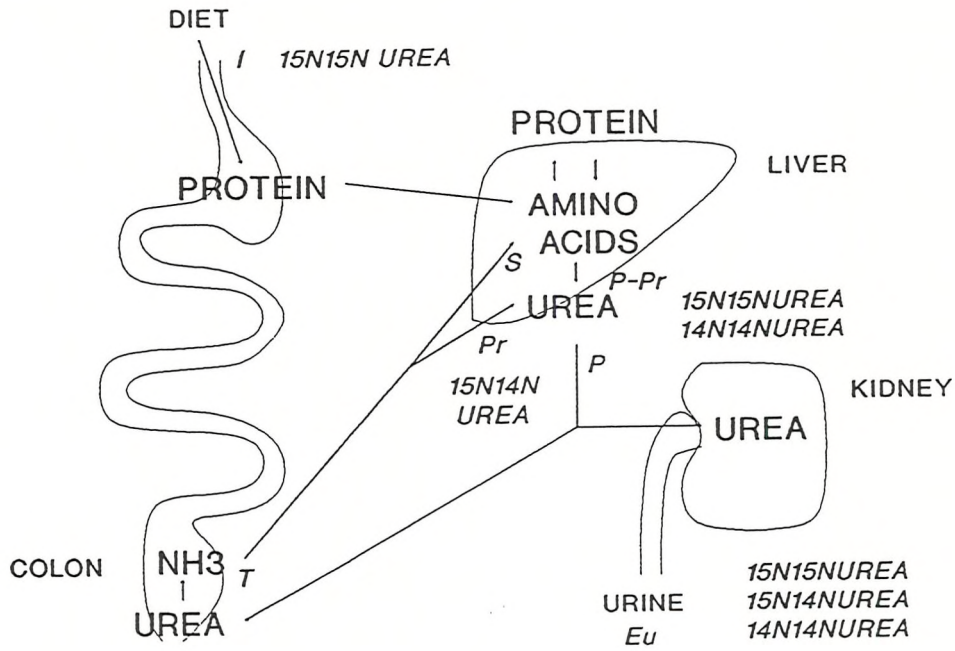
in the colonic microflora as administration of antibiotics eliminated the breakdown of urea (Walser & Bodenlos 1959, Long 1978). Walser estimated that 15-30% of urea synthesised is not excreted in the urine but degraded to ammonia and recycled.

The use of radioactive and stable isotopes was fundamental to the development of this theory. The classical studies by Schoenheimer opened the field for their use in more specific areas of protein metabolism. Studies are carried out with the assumption that stable isotopes are metabolised in the same way as the common isotope form. Walser determined that with a singly labelled isotope of urea (urea 29), it is impossible to distinguish between newly synthesised urea and the original dose, and hence it is also impossible to estimate the extent of urea- nitrogen reutilisation (Walser & Bodenlos 1959). Hence, urea metabolism was examined using a urea molecule in which both nitrogen atoms were ^{15}N. It was demonstrated that when nitrogen molecules are liberated by the reaction of urea with hypobromite, both atoms are derived from a single urea molecule and, by means of mass spectrometry, urea nitrogen molecules of mass 28, 29 and 30 can be distinguished (Walser 1954). Further, when isotopic nitrogen is used to label urea, the majority is formed as doubly labelled $^{15}N^{15}N$ urea (urea 30). In the body the vast majority (99.24%) of the urea is in the form of unlabelled urea (urea 28), with trace amounts of the other isotopic molecules. Therefore, an approach using this doubly labelled molecule to determine urea kinetics was established (Walser 1970).

Another approach was to label the carbon atom of the urea molecule (Walser & Bodenlos 1959, Jones *et al* 1969, Long *et al* 1978); however, these kinetic techniques using ^{13}C labelled urea were unclear as to the endogenous utilisation of urea as the design meant that the pathway of the carbon skeleton of the urea molecule was followed, not that of the nitrogen moiety. The carbon dioxide produced by the hydrolysis of urea is diluted in the large CO_2-bicarbonate pool and is difficult to trace.

The principles of urea kinetics are described in Figure 2.4, using the model of Jackson *et al* 1984. The urea-30 molecule is administered orally (I=ingestion) and passes into the body's urea pool where it mixes with unlabelled urea from endogenous production (P). The dilution of this doubly labelled isotope in the pool, ie. the ratio of $^{15}N^{15}N$ urea to $^{14}N^{14}N$ urea excreted in the urine gives us a value for production. Some label passes to the colon where bacteria hydrolyse it (T) to labelled ^{15}N ammonia. This reenters metabolism by one of four methods (Jackson 1993a); it can be absorbed as ammonia and fixed in the liver through amination of the non-essential amino acids, ie. as glutamate or glycine/serine; through wider transamination with the carbon skeleton of transaminating non-essential amino acids, ie. alanine, aspartate; through wider transamination with the carbon skeleton of transaminating amino acids; or by bacterial fixation as essential and non-essential amino acids which are then available to the host through absorption. Incorporation of the ^{15}N into amino acids is termed S for synthetic pathways. The labelled ammonia can also combine with an unlabelled ammonia molecule because of the small proportion of label in the body pool and form $^{15}N^{14}N$ urea (Pr). The dilution of this molecule shows us the amount of urea-nitrogen recycled in the colon which returns to urea formation.

Figure 2.4 The Principles of Urea Kinetics.



Two different approaches have arisen for studying urea kinetics. The single dose technique involves a protocol which administers the dose as a single bolus and follows isotopic excretion over a set period of time, usually 48 hours (Walser & Bodenlos 1959, Long *et al* 1978). This relies on the interpretation of a decay curve of isotopic enrichment and the estimation of urea pool size. As this method measures losses from the urea pool, it is susceptible to errors in the collection of urine; however, there are advantages in that the method is simple and non-invasive. A single dose method has

also been derived which measures cumulative excretion (Jackson *et al* 1993c). The cost of analysis is reduced with these methods as there are few samples and hence,it is possible to consider studies in larger groups.

The second technique is the intermittent dose method, whereby the labelled urea is not administered in a single dose but in several smaller doses so as to measure equilibrium in the urea pool (Picou & Phillips 1972). This allows a simpler mathematical model to be derived measuring urea production as the ratio of label to endogenous urea entering the pool. This does not require knowledge of the urea pool size or dependence on interpretation of a decay curve but is more labour intensive on human subjects.

The urea pool is large and slow to turnover and hence it takes time to reach the plateau of isotopic enrichment in urine necessary for calculations of urea kinetics. Experiment time to reach a steady state is reduced by giving a priming dose of isotope initially (Wolfe 1981). Hence, a two pool model was developed for measurement of urea kinetics in man using a non-invasive, primed, constant infusion of dose (Jackson *et al* 1984).

All of these methods are non-invasive as they involve the oral administration of dose. A major assumption underlying the reliable measurement of urea production is that the dose is absorbed intact and reaches the body pool of urea quantitatively. Use of oral methods, however, make the assumption that no hydrolysis of urea occurs in the upper gastrointestinal tract. Some individuals do not conform to normal urea kinetics and using a ^{13}C breath test, (Graham 1987), it can be seen that these people contain spiral gram negative bacteria attached to the gastric mucosa which have a high endogenous urease activity. Hence, the presence of organisms such as Helicobacter pylori means that it is not possible to be confident about the result of urea kinetics when an oral administration of isotope is used. In describing the

prime intermittent method, Jackson demonstrated that route of administration had no effect on isotope recovery with a direct comparison of oral, intravenous and intragastric dose administration (Jackson *et al* 1984). Further evidence that oral and intravenous administration of dose did not alter results was given (Hibbert 1992) and suitable limits in intra-individual were found (Hibbert & Jackson 1991) giving the conclusion that this method is reliable with good repeatability (Jahoor & Wolfe 1987). Recent studies have demonstrated comparable results using a lactose-ureide molecule which aims to deliver the isotope directly to the colon (Bundy *et al* 1995).

2.8 The Role of the Colon in Urea Nitrogen Salvage

One important consideration in the urea-nitrogen salvage system is the role of the colon and its permeability to the bacteria nitrogenous products. In many species, the large intestine provides a chamber for the final stages of digestion. However, this was not the traditional view in man where the colon was thought of as an organ that conserves salt and water, and disposes of waste material. The gut is inhabited by a colony of bacteria which are in intimate contact with their host and take part in numerous metabolic processes. The microflora consists of approximately 400 species, the composition of which depends mainly on diet (Hill & Draser 1975). Intestinal bacteria produce ammonia by deamination of amino acids, from dietary residues or intestinal secretions and cells with the principle route of ammonia production suggested as urea hydrolysis to ammonia and carbonic acid by bacterial ureases in the colon (Summerskill & Wolpert 1970, Cummings & MacFarlane 1991).

Between 6-9g of urea, about 20% of urea production, is catabolized in the colon each day. Hardly any urea is detectable in the faeces and only 1-3mmol of ammonia; virtually

all of the urea entering the colon is hydrolysed and the metabolic products absorbed (Wrong *et al* 1965). Urea hydrolysis occurs in a juxtamucosal situation, as very little colonic mucosal urease activity is present in man (Aoyagi *et al* 1966) and intravenously administered urea is metabolised more rapidly than urea perfused through the colonic lumen (Wolpert *et al* 1971).

One problem to the theory of urea kinetics was that it was unclear how the urea passed into the colon for hydrolysis. A small proportion (10%) of urea in the colon can be said to derive from the ileum, leading to the conclusion that substantial quantities must diffuse into the colon from the blood (Gibson *et al* 1976, Chadwick *et al* 1977). However, investigators thought that the colon was impermeable to urea diffusion (Levitan & Billich 1968, Wolpert *et al* 1971). This was based on the fact that concentrations of urea in the large intestine are relatively low. It has been demonstrated, however, that when di-labelled urea was placed into the colon, a significant proportion of the dose was recovered as urea-30 in the urine, showing that the colon was permeable to the intact urea molecule (Moran & Jackson 1990a, 1990b). It was seen in very early samples because of rapid diffusion, suggesting why earlier perfusion studies had reaches a different conclusion. Approximately 15% of the dose was found in the urine as urea-29 which represented urea that was hydrolysed and absorbed as ammonia. Less than 4% was found in stool and, hence, the majority could not be accounted for and was presumed to have entered the metabolic nitrogen pool, although juxtaluminal hydrolysis and retention cannot be discounted.

Ammonia is potentially harmful to the host and is rapidly absorbed from the gut and detoxified by urea formation in the liver. Ammonia concentrations in the gut lumen can be reduced by active carbohydrate fermentation which stimulates the bacterial requirement for nitrogen due to increased growth in

bacterial numbers. Nitrogen is incorporated into bacterial amino acids which can be absorbed across the mucosa of the colon wall and are retained in the protein pool at an efficiency of approximately 90% (Heine *et al* 1987). This suggests that the colon wall is permeable to intact amino acids in infants but, in adults, it was suggested that the large intestine lacks the absorption mechanism (Wrong *et al* 1981) and the free amino acids are fully utilised by the bacteria (Macfarlane & Allison 1986). There is now evidence, however, for a dipeptide transporter in the colon and this could provide a method for bacterially produced amino acids to be available to the host (Fei *et al* 1994).

2.9 The Functional Significance of Urea Nitrogen Salvage

At the time when recommendations for protein intakes were debated (FAO 1985), it was considered that evidence for urea salvage playing a significant role in nitrogen metabolism was not strong. Even recently, the following statement was made:

> "our findings lead us to conclude that the major fate of the ammonia that is released via urea hydrolysis is its return to the urea cycle, effectively resulting in a 'futile' cycle, although this is not to rule out a physiological purpose of such a process in certain circumstance." (Young 1994).

However, there is unmistakable evidence to demonstrate that urea nitrogen is salvaged and made available to metabolism under normal physiological conditions (Jackson *et al* 1984, Hibbert & Jackson 1991, Jackson 1993c). Urea nitrogen salvage becomes a fundamental mechanism for maintaining nitrogen balance in situations of metabolic stress. For a fixed demand, salvage increases as intake falls and for a fixed intake, salvage increases as demand increases (Jackson & Wootton 1990).

One of the most important conditions which results in an

increased demand for nitrogen is growth. The most rapid period of growth in an individual's lifetime is in utero. Demand for nitrogen is not distributed evenly throughout pregnancy; it increases in relation to the accretion of placental and foetal tissues, as well as the deposition of maternal tissue which may be utilised in later pregnancy or lactation (deBenoist 1985). It appears that the increased demand for nitrogen is met by an increase in urea salvage in the colon (Forrester *et al* 1994, McClelland 1994).

Demand for nitrogen is also great in the newborn but surprisingly, the nitrogen content of human milk is low and not all of the protein in milk is digested or absorbed. About 25% of nitrogen in human milk is non-proteinous, the majority being in the form of urea. It also appears that the foetus and infant have much increased demands for non-essential nitrogen as glycine (Widdowson *et al* 1979), which ties in with the demonstration that nitrogen balance could be restored by addition of glycine or urea to a low protein diet in infants (Snyderman *et al* 1962). The mechanism by which infants deal with this increased demand is to have a high level of urea salvage in the colon (Jackson 1989). Studies have shown that, in infants, approximately 75% of urea production in the body is hydrolysed in the colon and 90% of this nitrogen is retained in the body pool (Wheeler *et al* 1991, Steinbrecher 1994). Another reason suggested for an enhancement in urea salvage is that in the foetus urea production appears to be driven by the oxidation of amino acids as an energy source (Jackson 1994a). Whereas the foetus can clear the large amounts of urea formed by diffusion across the placenta, in the newborn urea salvage is an important mechanism for handling urea production without overwhelming renal function.

Urea nitrogen recycling is also important during childhood growth and, in particular, in catch-up growth following malnutrition or disease (Jackson *et al* 1990a, Jackson 1990b, Jackson & Forrester 1993b) where demand for nitrogen is increased.

As well as growth, the other situation of increased nitrogen demand is during pathological insult. Urea salvage increases in the immediate post-operative period, and following major surgery a convalescent increase in salvage may last for up to six months (Jackson & Moran 1992). In homozygous sickle cell disease there is a marked difference in urea kinetics, probably due to an increased demand for red blood cell synthesis (Jackson *et al* 1988).

Another case where colonic salvaging is important is chronic renal failure. It has been shown in renal failure that urea recycling is increased compared to in normal individuals (Giordano 1963); ie. these patients seem able to utilise the increased circulating urea whilst on a low protein intake (Giovannetti & Maggoire 1964, Richards 1967, Varcoe *et al* 1976, Hou *et al* 1985, Ihle *et al* 1989). One possible solution to the damage caused by the oncotic pressure of protein on the glomerulus would be to feed diets restricted in protein (Giovannetti & Maggoire 1964, Brenner *et al* 1982, Rosman *et al* 1984) but containing the keto analogues of essential amino acids which could combine with the recycled nitrogen to form amino acids and further nitrogen in a non-proteinous form (Wrong *et al* 1970). Hence, careful nutritional management (Kopple 1978, Brocklebank & Wolfe 1993) and nitrogen recycling in the colon become fundamental components in the treatment of chronic renal disease.

However, the most important contribution of nitrogen recycling appears to be a fundamental aspect of the body's adaptation mechanism to differing nitrogen intakes.

2.10 Adaptation to Changing Nitrogen Supply

The changes in urea nitrogen recycling quoted above show examples of the body's mechanism of dealing with conditions where demand for nitrogen is increased; however, this system is also vitally important when the supply of nitrogen for a given demand is altered.

Requirements for total nitrogen can be influenced by the process of adaptation. If nitrogen intake is reduced below the natural requirement level, it does not mean that the individual dies; it would be expected that in time they would arrive at a new stable state but with a smaller lean body mass. Adaptation represents a steady state which can be maintained and this implies a long duration in time. This distinguishes an adaptation from a response which is immediate and often short lived, such as homeostatic controls. This is often an on/off response, whereas adaptation is a resetting of metabolic function. Adaptation is also reversible with the possibility, when conditions change, of moving from one point to another within the preferred range. If lowering of intake is too great, this process will not be established and death will ensue; hence, there is a limit to adaptation.

Adaptation is generally thought of as a process which has a successful outcome and is beneficial to the organism; however, there should be no assumption that all the functions of the individual are maintained. Beyond the range of homeostasis, adaptation involves a necessary selection among body functions to preserve those that are most essential. As intake falls there is a progressive sacrificing of function and a reduction in homeostatic reserve. The cost is an increasingly stressed metabolic state with a progressive decrease in the ability to cope with any challenge from the environment. The risk of disease and inability to maintain function increases; hence the mobilisation of the labile protein pool to allow the body to adapt to decreased intake is not without functional significance.

It is often necessary to distinguish between different levels of adaptation; genetic, physiological and behavioral. Mechanisms of adaptation occur at all three levels. In the case of nitrogen metabolism, adaptation can occur at the level of translation by phosphorylating initiation factors and at a physiological level with hormonal control altering protein turnover. In the context of this study, the body can adapt to altered nitrogen intake by conserving nitrogen losses from the

body, ie. by decreased urinary excretion, and by increasing the recycling of nitrogen in the colon.

Adaptation to varying nitrogen intake alters tissue protein and amino acid metabolism to produce more efficient utilisation of endogenous amino acids (Young *et al* 1985, 1990). These changes involve reduction of urea cycle enzymes (Schimke 1962) and of enzymes associated with degradation of amino acids (Harper 1968) and with a shift in the distribution of body protein synthesis (Waterlow 1968). The physical indication of these processes occurs at the level of nitrogen excretion.

It has long been recognised that the amount of nitrogen lost in the urine reflects the nitrogen balance of the subject under study. Nitrogen excretion falls exponentially with a reduction in nitrogen intake until a new equilibrium is reached after 4-5 days (Gopolan & Narasinga Roa 1966, Rand *et al* 1976, Price *et al* 1994). Waterlow developed a model based on a single pool of metabolic nitrogen receiving input from diet and protein catabolism, while nitrogen is lost by excretion or protein synthesis (Waterlow 1968). Using labelled amino acids to look at whether the turnover of body protein is linked to dietary protein or redistribution of the flow of amino nitrogen is preserved, he showed that the response is due to a reduction in excretion of nitrogen accompanied by a greater utilisation of amino acids for protein synthesis. Total protein turnover patterns became altered with peripheral tissues decreasing their metabolic demand to enable the restricted nitrogen supplies to be channelled to vital organs such as the liver. This is a phenomenon seen in many systems of malnutrition. Faecal nitrogen output appears to be unaffected by nitrogen intake (Weller *et al* 1971, Hounslow 1995) and although dermal losses may change in proportion to intake (Calloway *et al* 1971), compared to urinary excretion these losses are insignificant.

The mechanism by which urinary nitrogen output is adjusted with alterations in intake partly involves the control urea production by the urea cycle enzymes. The enzymes responsible

for this system are compartmentalised in the hepatocyte (Watford 1991) and open to regulation. In mammalian liver, the cycle is subject to both short-term (changes in specific activity of existing enzyme protein) and long-term regulation (changes in the amount of enzyme protein). The first real evidence of this came from the experiments by Folin who, by feeding himself a low-protein diet of arrowroot starch and cream, showed that urea production was related to dietary protein load (Folin 1905). This was followed in the 1930's by the demonstration that arginase activity varied with dietary protein level and later, with the co-ordinate regulation of the five urea cycle enzymes in response to dietary protein intake and during starvation (Schimke 1962). Although changes in the activity of all five enzymes appear to be co-ordinately regulated, the molecular mechanisms are not always the same for each enzyme. Regulation occurs at one or more of a number of sites, from protein synthesis and degradation to changes in gene transcription. In the short term, Carbomoyl phosphate synthetase is allosterically activated by N-acetylglutamate which is known to be increased under conditions of raised urea production (Shigesada *et al* 1978). Also the concentration of ornithine in the mitochondria may play an important role in the regulation of carbamoyl phosphate synthetase activity and flux through the cycle (Lund & Wiggins 1986). The system is not fully understood, however, as it has been shown that urea production can increase without a rise in the amounts of urea cycle enzymes, because the potential capacity of the enzyme system is not normally fully utilised (Das & Waterlow 1974). It was proposed that this system does not work in isolation but nitrogen supply will also affect the activity of enzymes involved in protein synthesis. Cohen also made the point that endocrine control mechanisms play a role in the regulation of protein metabolism and can more specifically affect the urea cycle enzymes (Cohen *et al* 1981).

Hence, changes in urea cycle enzymes and amino acid metabolism control the rate of urea production with altered nitrogen intake; however, adaptation can also occur by altering

the proportions of urea production which are excreted or recycled in the colon.

2.11 Adaptation by Urea Nitrogen Salvage

So how do the colonic bacteria contribute to the adaptive response to changes in protein intake? The answer was first alluded to in studies of uraemic patients, a group who have long term lowered protein intakes. Due to limitations regarding the nitrogen balance technique, the incorporation of ^{15}N from urea into amino acids was investigated in patients with chronic renal failure (Giordano *et al* 1968). It was found that this incorporation into non-essential amino acids occurred to a greater extent in the uraemic patient; in fact, up to three times more. Suppression of intestinal microflora with antibiotics greatly reduced urea utilisation, suggesting a role for the colonic microflora. Utilisation of urea nitrogen for albumin synthesis was found to be higher in uraemic patients than in normal subjects, and also in patients on a lower protein intake compared to those on a normal intake (Varcoe *et al* 1976).

Further evidence for the importance of urea salvage in lowered nitrogen intake came from examination of urea kinetics in malnourished children in the Tropical Metabolism Research Unit, Jamaica. It was found that on a low protein diet, the rate of urea production fell (Picou & Phillips 1972). The amount of urea excreted in the urine was much reduced, meaning that the amount of urea nitrogen utilised for protein metabolism was increased to 69%. It was also found that malnourished children were able to adapt to a reduced protein intake as easily as the well nourished child. It was found that during catch-up growth, nitrogen is a limiting factor and urea recycling alters to accommodate this (Jackson *et al* 1990a). Once again, the pattern of increased urea hydrolysis and reincorporation of this nitrogen into the body pool occurs when nitrogen intake is reduced. Hence, the approach of urea

kinetics could be useful in defining the adequacy of nitrogen in the diet.

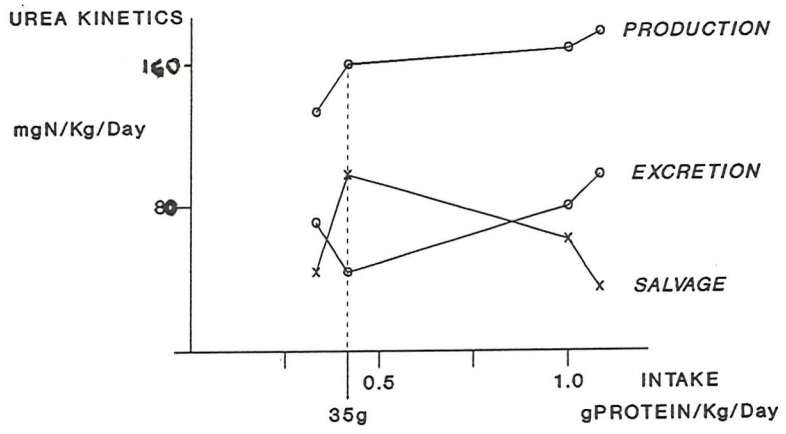
The same responses are seen in normal adults. Urea metabolism was followed during low protein intake in man and dog using the ^{15}N isotope and it was concluded that although urea production is decreased on low protein diets, the rate of urea degradation is sustained (Murdaugh 1970). In the same year, utilisation of exogenous urea was found to improve nitrogen balance in malnourished subjects (Tripathy *et al* 1970), tying in with the experimental findings of Kies (Kies *et al* 1965b, 1965c, 1978). The fact that this response was suppressed by antibiotics was an indication that the colonic bacteria were involved in this utilisation. Following these studies, the relationship of urea production to dietary intake of nitrogen was examined (Jackson *et al* 1981a). It was shown that on a normal diet, urea nitrogen production was greater than nitrogen provided in the diet; the difference being obtained from recycling to urea after colonic hydrolysis. As nitrogen intake fell there was a decrease in urea production, but a larger proportion is degraded in the bowel and reutilised into metabolism rather than urea synthesis.

A great body of evidence to support this theory that urea salvaging is increasingly important when protein intakes are reduced comes from numerous Japanese studies on the highlanders of Papua New Guinea (Koishi *et al* 1990). These people have adapted to a long term reduced protein intake of less than 40g/day. Three possible mechanisms were suggested; a slow rate of growth and small physique, a high level of urea nitrogen and fixation of gaseous nitrogen in the intestinal flora. The PNG highlanders were, in fact, 10% shorter in stature and 5% lighter in weight than people living in a rural Japanese community (Ueda *et al* 1979). It was suggested that the PNG highlanders may be capable of gaseous nitrogen fixation (Oomen 1970), similar to that found in leguminous plants, but no bacteria capable of this were found in faeces. However, an

increased number of bacteria with a urease activity were found and studies using labelled urea have shown that PNG highlanders possess a more efficient salvaging system to reutilise urea nitrogen for protein synthesis (Miyoshi *et al* 1986). Normal japanese controls only utilised significant amounts of urea nitrogen when they consumed a low protein diet.

Two recent studies from Jackson's research group have looked more closely at the adaptive response to a low protein diet (Langran *et al* 1992, Danielsen & Jackson 1992). Figure 2.5, taken from Jackson 1992, summarises these two studies and shows the reciprical relationship between urea excretion and salvage with production maintained at a constant level as the intake of dietary nitrogen falls.

Figure 2.5 Urea Kinetics over a range of Protein Intakes.



It was found that on a 70-74g protein/day diet, a reflection of the average intake of males in the U.K, the majority of urea produced was excreted in the urine whilst 40% was salvaged in the colon. However, when intake was lowered to 35g protein/day, only 36% of the urea produced was excreted with the majority, 64%, being salvaged. Hence, as protein intake falls, urea salvage increases to recycle the limited nitrogen available, and therefore excretion of urea falls. This data reinforced the conclusions drawn from earlier studies that urea nitrogen utilisation in the colon is an integral part of the process of adaptation. The sum of intake and salvaged nitrogen did not alter, implying quantitative control over urea salvaging rates.

Nitrogen balance was no longer maintained when the intake was reduced to 30g protein\day as this adaptive system failed. Urea production and salvaging both fell, with a net increase in nitrogen loss in the urine. These changes in urea kinetics can be used to determine that the minimum physiological requirement for protein in the normal adult male lies between 30-35g/day (4.8-5.6gN/day). Between these intakes, a switch point occurs which enables the body to maintain nitrogen balance despite low protein intakes by nitrogen recycling in the colon. However, the factors which control this switch point and the rate of urea salvage over a range of intakes remain unclear.

2.12 Control of Urea Nitrogen Salvage

Urea salvaging appears to have all the characteristics of a controlled process, but the nature of the control still needs to be resolved. It is clear that salvage is not simply a clearance phenomenon determined by plasma concentration of urea (Walser 1974, Jackson 1995). The extent of salvage appears to relate to a balance between the metabolic demand for nitrogen and the provision of protein or nitrogen in the diet. However, this determinant does not work in isolation from the body's metabolism.

The source of nitrogen may be important. The nitrogen balance studies of Kies demonstrated that the varying nitrogen source had different effects on nitrogen balance. Protein sources do not appear to influence urea salvage, as when comparable amounts of protein are fed from vegetable sources there is no change in nitrogen balance (Gattas *et al* 1990) or urea salvage (Bundy *et al* 1994).

The interactions of protein and energy metabolism are well established with excess energy intake enhancing nitrogen balance (Calloway 1975, Motil *et al* 1981a), so it is not unrealistic to assume that urea salvage is influenced by energy balance. Indeed, faecal output was reduced on a low energy intake, suggesting a modulation of gastrointestinal function (Kennedy *et al* 1990). The utilisation of non-essential nitrogen to improve nitrogen balance is enhanced by raising calorie intake (Anderson *et al* 1969) and shortening time required to adapt to a lowered protein intake (Inoue *et al* 1973). Demand for energy has also been shown to influence urea salvage in that salvage may be related to lean body mass and, hence, basal metabolic rate (Child 1995). Exercise does not stimulate urea production but may accelerate the reincorporation of urea nitrogen back into body protein (Carraro *et al* 1993).

Other nutrients may influence the rate of urea salvage but there have been few experiments in this area. When nitrogen and energy intakes are low, urea kinetics can be influenced by the relative proportion of fat and carbohydrate in the diet (Jackson *et al* 1990a). However, complex carbohydrates do not change urea salvage on low protein intakes when energy is not limited (Langran *et al* 1992, Doherty & Jackson 1992). Hence, whether fat and carbohydrate metabolism imposes any control over urea salvage remains to be shown.

Urea metabolism is not purely a mechanism involved in amino acid turnover. Urea has other roles in the body and these may influence the rate of urea hydrolysed in the colon. Fluctuations in acid-base balance may alter the proportion of urea passing the colon as the formation of urea from two

ammonia molecules and a bicarbonate and its subsequent excretion is central to the control of pH (Atkinson & Bourke 1984, Haussinger 1990).Urea also plays an important role in the conservation of body water. Transport of urea across the nephron, the 'counter current mechanism', is essential for the ability of the kidney to concentrate urine. Urea transport in the collecting duct is stimulated by vasopressin regulated urea transporters (Gillin & Sands 1993). Vasopressin, or antidiuretic hormone, stimulates urea permeability by increasing the number of functional urea transporters. These transporters have also been found in the colon (You *et al* 1993), an organ of water reabsorption, and hence urea concentrations in the colon may play a role in fluid balance.

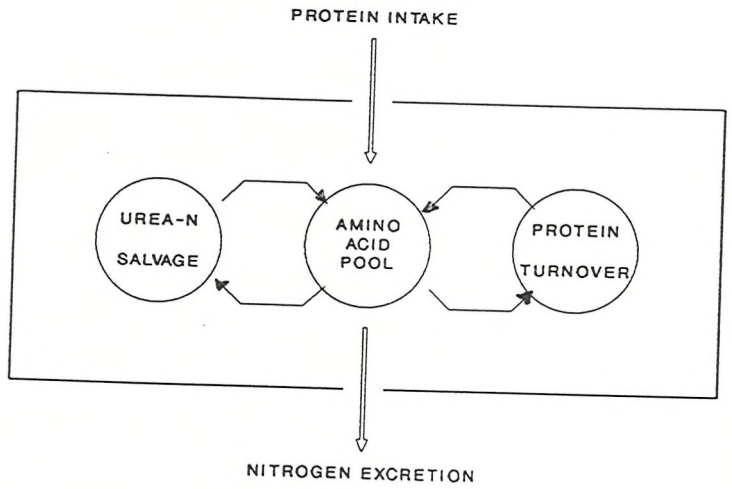
Protein metabolism is also influenced by diurnal and circadian rhythms. It has been demonstrated that protein turnover has a diurnal cycle with the rate of protein synthesis being lower during the night (Garlick *et al* 1980, Grove 1993) and a diurnal variation of urea excretion (Steffee *et al* 1981). It has been calculated that urea salvage may also be subject to changes between day and night (Jackson 1994b). Changes in protein turnover (Grove & Jackson 1992) occur during the menstrual cycle. Urea salvage only appears to be altered through the menstrual cycle in women taking oral contraceptives (McClelland 1994).

Hence, the rate at which urea nitrogen is salvaged is determined by the dietary intake of protein in relation to the metabolic demand for protein synthesis and net protein accretion. For a fixed demand, salvage increases as intake falls. For a fixed intake, salvage increases as the demand increases. This is a fundamental aspect of the body's ability to adapt to changing nutritional intakes. However, this process of adaptation is one of the reasons why understanding of requirement levels in human populations remains unclear.

2.13 Aims of this Thesis

The whole question of protein, and hence nitrogen, requirements remains unresolved and under constant debate. The problem will only be resolved with further understanding of the mechanisms of nitrogen metabolism. A full consideration of the adequacy of diet in terms of nitrogen requires that there is a clear understanding of the interactions of each of the three aspects of the model of nitrogen, protein and amino acid metabolism (Figure 2.6); external balance and the two internal cycles, protein turnover and urea nitrogen salvage.

Figure 2.6 The Interactions of Nitrogen Metabolism.



This thesis will attempt to address some of the important issues in the debate for nitrogen requirements by looking at factors influencing the recycling of nitrogen in the colon.

Although still under constant criticism, there is now undeniable evidence for this system and its fundamental importance in the physiology and nutrition of individuals.
By examining the response of the colonic bacteria to low protein intakes supplemented with sources of non-essential nitrogen, the relative contributions of protein and total nitrogen intake can be assessed. By drawing together the two lines of research it may be possible to examine the essentiality of non-essential nitrogen from various sources using the technique of urea kinetics, ie. is the improvement in nitrogen balance when a low protein diet is supplemented with non-essential nitrogen due to the 'switching on' of urea salvage mechanism? This will hopefully further the insight into nitrogen and protein nutrition and continue the engaging debate.

CHAPTER 3

METHODOLOGY

3.1 The Prime Intermittent Dose Model of Urea Kinetics

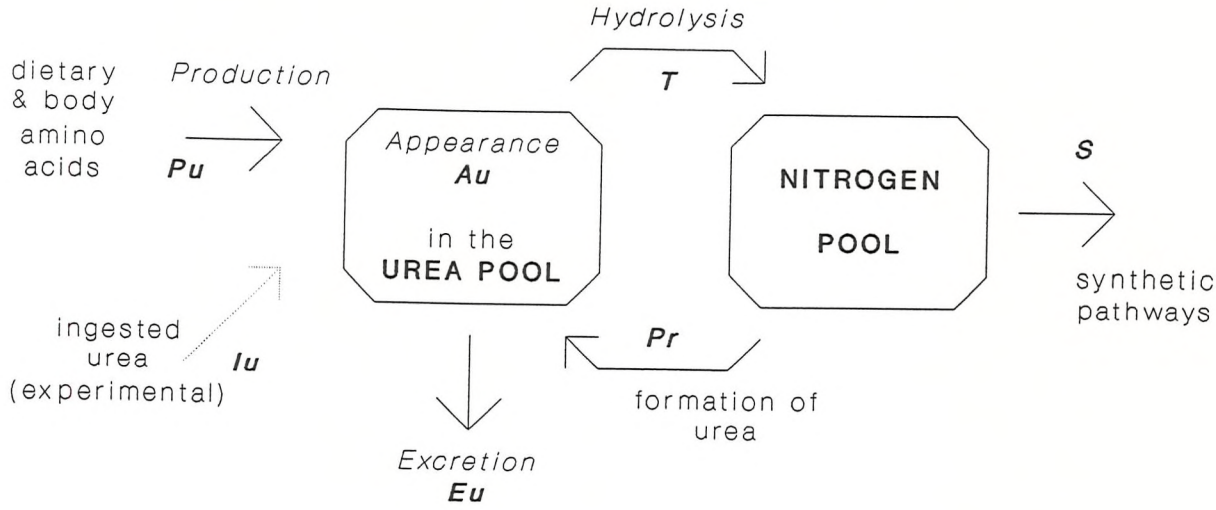
Urea is the end product of nitrogen metabolism and hence, urea excretion is frequently used as a measure of net protein and amino acid catabolism. This approach applies the assumption that urinary urea excretion directly reflects endogenous urea production. However, a proportion of urea produced passes to the colon and undergoes bacterial hydrolysis so a method which measures urea production directly is more appropriate when considering the components of nitrogen metabolism.

Urea kinetics were calculated using an adaptation of the model of Jackson *et al* 1984 (Figure 3.1). The model assumes the existence of two pools, a urea pool and a nitrogen pool, within which nitrogen derived from degraded urea moves freely. It assumes a metabolic steady state, ie. constant pool size with constant input to and output from the pool.

Urea can enter the urea pool from one of three inputs. The first is via amino acid oxidation. The ammonia produced is detoxified in the liver by combining with CO_2 to form a urea molecule via the urea cycle. In a number of supplemental studies in this thesis, non-essential nitrogen is ingested as urea (Iu). Hence, the appearance (Au) of urea in the body pool is taken to be the sum of ingested urea and that produced endogenously, ie. Au=Iu+Pu. A third source of urea for the urea pool is the urea formed from nitrogen recycled in the colon (Pr). As will be described, the use of di-labelled urea allows, urea produced in this way to be measured separately from endogenous production.

Urea in the pool has two possible fates. It can pass to the colon, be hydrolysed by bacterial urease and the nitrogen incorporated into the nitrogen pool, or, is excreted in the urine.

Figure 3.1 The Two Pool Model of Urea Kinetics



The nitrogen produced from hydrolysed urea can leave the nitrogen pool into synthetic pathways (S), for example incorporation into amino acids, or is used to form urea (Pr).

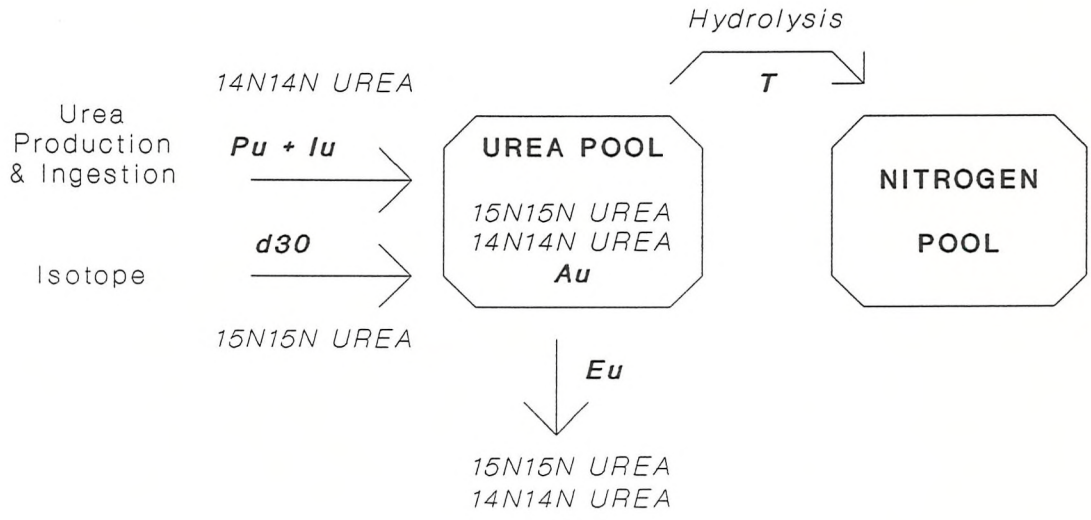
In order to measure the components of this model, a prime intermittent dose of the stable isotope $^{15}N^{15}N$ urea was administered.

^{15}N is a stable isotope with an extra neutron in the nucleus. It does not decompose to emit radioactive particles and is present in the atmosphere as 0.37% of nitrogen. ^{15}N is usually expressed as atoms percent excess which is a ratio of the isotopic content of a sample above the ratio of natural abundance. As background levels are constant, delta values are not widely used as they represent a ratio relative to a defined

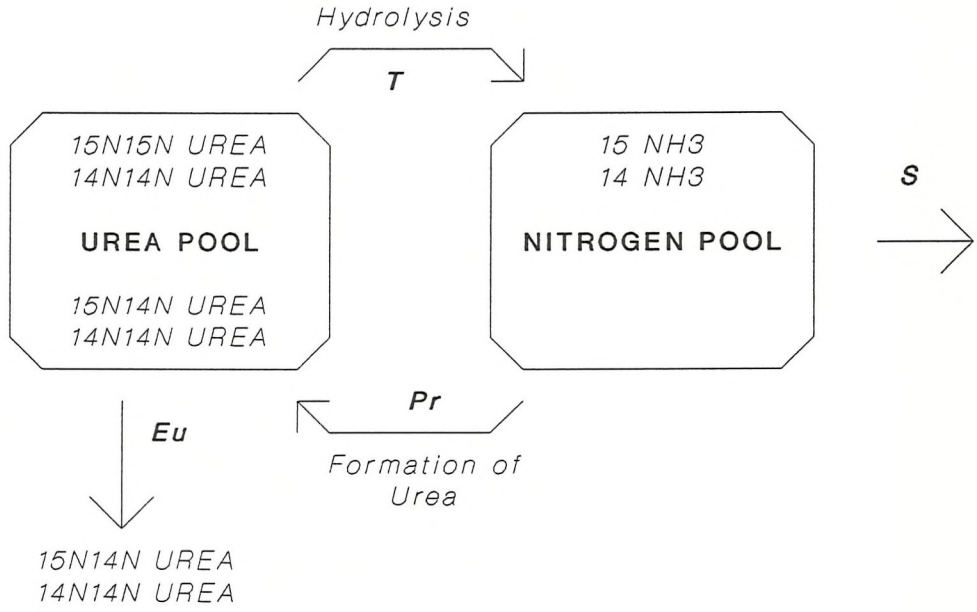
reference value and have been most widely used when considering the ^{13}C isotope. In this model of urea kinetics, the ratio of $^{15}N^{15}N$ urea:$^{14}N^{14}N$ urea and $^{15}N^{14}N$ urea:$^{14}N^{14}N$ urea are determined by Isotope Ratio Mass Spectrometry.

Urea kinetics have been measured using urea molecules labelled with ^{13}C and ^{18}O to measure urea production and follow the fate of these molecules after urea hydrolysis (Long 1978, Matthews & Downey 1984). When carbon labelling has been used, the values for production appear higher and this isotope gives information on formation and excretion only. Therefore, it is more appropriate to follow the fate of nitrogen. If a single ^{15}N label is used, it is not possible to distinguish between newly endogenously synthesised urea and the administered label. Hence an approach was developed in which a doubly labelled urea molecule is used (Picou & Phillips 1972, Wolfe 1981, Jackson 1984).

In the method of constant infusion, the $^{15}N^{15}N$ urea molecule is introduced to the urea pool and the ratio between the labelled urea and the non-labelled urea approaches a constant which is identified as a plateau of urinary enrichment of $^{15}N^{15}N$ urea to $^{14}N^{14}N$ urea.



Measurements of urinary urea enrichment assume that the dose of label is handled in the same manner to that endogenously produced. The ratio gives a direct measure of endogenous urea production (and ingested urea if supplemented) as the ratio of the two urea species in the pool will be equal to the ratio of input to the pool from endogenous production to the administered dose. If urea excretion in urine is known, then the difference between the measured urea production and that excreted is assumed to be the urea passing to the colon.



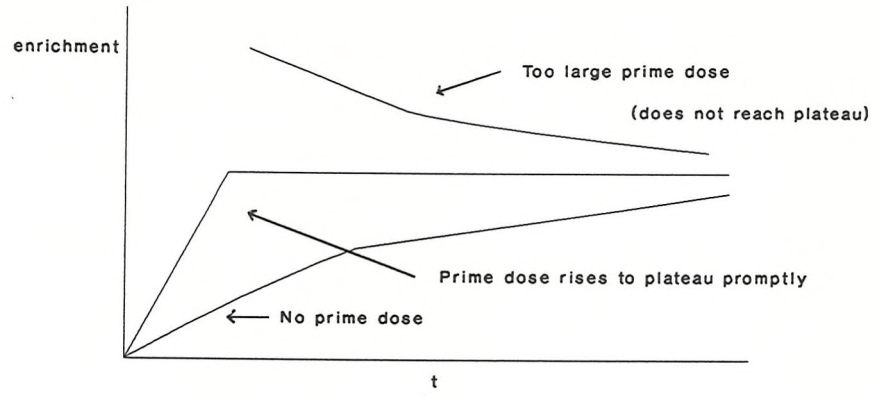
The nitrogen produced from urea hydrolysis enters the body's nitrogen pool. The rate of urea formed from a $^{15}NH_3$ joining with an unlabelled NH_3 molecule from the body pool to form urea (Pr) can be measured directly by the ratio of $^{15}N^{14}N$ urea to $^{14}N^{14}N$ urea in the urine and hence, the proportion of recycled nitrogen entering synthetic pathways can be derived; S=T-Pr.

A further assumption of the model is that the dose of $^{15}N^{15}N$ urea is presumed to enter the large bowel intact after being absorbed in the upper gastrointestinal tract and entering the colon by diffusing through the colonic wall. Evidence from different routes of administration, ie oral or intravenous, give comparable results (Hibbert *et al* 1992). However, in individuals with *Helicobacter pylori* infection, part of the label may be hydrolysed in the stomach and the model for urea kinetics would not hold under these circumstances. In order to have confidence this is not occurring, a ^{13}C breath test can be performed (Graham 1987). Another indication would be an unrealistically high rate of $^{15}N^{14}N$ urea excretion in the urine (Hibbert *et al* 1992). Studies are currently being carried out using lactose ureide to deliver the label to the colon directly (Bundy 1995).

The protocol in this thesis uses a prime intermittent dose technique administered orally so as to be non-invasive. A priming dose is given in order to shorten the time taken to reach isotopic equilibrium in the pool.

Urea has a slow turnover rate and hence, when a priming dose of tracer is administered into the large substrate pool, the priming dose will disappear slowly (Matthews & Downey 1984). Therefore, if the size of the dose is incorrect, the tracer enrichment plateau may artificially reflect the priming dose and not the true metabolic production rate. Calculations are based on the single pool model of Shipley and Clarke (1972). This states that turnover rate is equivalent to the input-output ratio of material through a pool in a steady state system. When an isotope is infused continually into an unlabelled substrate pool, the relative concentration of the isotope to unlabelled substrate is the same as the proportion of infused tracer to the endogenous input of unlabelled substrate in the pool and as long as input rates remain constant, the substrate enrichment will reach a steady state and a plateau of enrichment is reached. The effect of a priming dose is to reach plateau enrichment in a shortened time.

Figure 3.2 The Effect of a Priming Dose



However, care must be taken when applying this simple model to complex biological systems. The requirement for accuracy in prime doses can be met experimentally. It is possible to determine the appropriate priming dose (Wolfe 1992) by estimation of body pool size and rate of appearance. A pilot study can then be performed giving a bolus dose of the label and examining the decay curve. The goal of a prime dose is to create the situation in which the sum of the decline in enrichment resulting from the bolus prime dose and the rise in enrichment from continuous infusion are equal to the ultimate plateau of enrichment. In the protocol used in this thesis, a prime dose equivalent to 15 hours continuous infusion was given, as used previously (McClelland 1994).

3.2 Determination of Ammonia and Urea Nitrogen

3.2.1 Ammonia Nitrogen in Urine

Ammonia in the urine was determined using the Berthelot method (Kaplan 1965). The concentration of ammonia is estimated by reaction with phenol and alkaline hypochlorite to form p-quinone chloroimine. The p-quinone imine reacts with another molecule of phenol to form indophenol which then dissolves to yield a blue indophenol dye. The reaction is catalysed by sodium nitroprusside.

The reaction was adapted to take place in a microtitre plate, enabling it to be read in a biotek EL340 microplate biokinetics reader. Hence it was necessary to scale down from a volume of 10ml to 200µl.
After calculating volumes and testing varying dilutions to ensure that there was sufficient reagent to complete the reaction, the results were found to be comparable to those obtained using the test tube method currently used. The time course of the reaction was followed and it was found that after 20 minutes incubation at 37°C, the reaction was completed.
Coefficients of variation were estimated by running a plate with 12 wells of each sample and was found that intra individual variation had a mean CV of 7.28%. Standard curves were also very repeatable with a mean r value of 0.997. The reaction also used far less reagent which improved accuracy and reduced cost. Many more samples could be assayed at once.

The method was as follows:-

1. Urine samples and standards (500mg NH_4-N\100ml diluted to form four standards of 1.0, 2.0, 3.0, 4.0µg NH_3-N\10µl) were diluted 1:10.

2. 10µl of these diluted samples and standards were taken and added to individual wells of a microtitre plate, each sample in triplicate.
3. Then 100µl of phenol nitroprusside solution (PNP) was added to each well.
   PNP = 25g phenol in 250ml deionised water plus 125mg sodium nitroprusside in 50ml deionised water (dissolved separately) made up to 0.5 litres.
4. 100µl alkaline hypochlorite solution was then added to each well.
   HYPO = 12.5 mg sodium hydroxide pellets in 400ml deionised water plus 20ml domestos household bleach (5%NaOCl) made up to 0.5 litres.
5. The microtitre plate was then incubated at 37°C for 20 minutes in the plate reader which then read the absorbance of each well at 550nm. From the standard curve, the concentration of the ammonia N in the urine was then calculated.

3.2.2 Urea Nitrogen in Urine

Urea in the urine is hydrolysed by the specific enzyme urease and is converted to ammonia and carbon dioxide, with carbamic acid as a probable intermediate stage. The reaction is buffered with EDTA which also serves to chelate any heavy metal ions that might otherwise inactivate the urease. The ammonia is estimated by the Berthelot reaction as already described in section 3.2.1. The final dilution of the urine sample is so great that precipitation of proteins is unnecessary.

This reaction was again adapted for the biotek plate reader. However, it was not possible to simply work out volumes necessary from 10ml to 200µl as the enzyme reaction did not follow expected patterns. Several dilutions of stock enzyme were tested but no pattern in the results allowed calculation

of a suitable concentration. This may have been due to the fact that the working enzyme contained EDTA whereas the stock enzyme does not. Hence the first part of the reaction, the hydrolysis of urea, was still carried out in a test tube. In future, work needs to be carried out to determine whether the total reaction can be converted to the microtitre plate.

The method was as follows:-

1. Samples were diluted 1:20. Standards of 2.5, 5.0, 7.5, 10.0µg urea N/25µl were made from a solution of 500mg urea N/100ml with 100mg sodium azide as a preservative.
2. 25µl of standards and diluted urine were then added to labelled test tubes in duplicate (standards in triplicate).
3. To each tube 1ml working urease was added and incubated at 37°C for 20 minutes.

   STOCK UREASE - (30units\ml) 200mg Jack Bean Urease Type III in 100ml deionised water and 100ml glycerol.

   WORKING UREASE - 1ml stock urease to 100ml EDTA buffer (10.og Na_2-EDTA in 100ml deionised water at pH 6.5.
4. 20µl of each solution in the test tubes was added to wells in the microtitre plate and reacted with 100µl PNP and 100µl alkaline hypochlorite. The plate was then incubated at 37_oC for 20 minutes and then the absorbance read at 550nm. The concentration of urea N in the urine was then calculated by subtracting the ammonia N results from the ammonia N obtained from urea hydrolysis.

3.2.3 Urea Nitrogen in Plasma

Urea was determined in plasma taken from subjects and treated in the following way:

1. 20mls of blood was taken from the subject by venesection of the ante cubital vein.
2. The blood was placed into a universal containing 2ml of EDTA buffer (o.1M EDTA + 0.4M saline at pH7.4) and then spun in a centrifuge at 4°C at 3000rpm for 10 minutes to separate the plasma from red cells.
3. The plasma was then pipetted as 1ml alliquots into cyrovials containing 0.2ml of an enzyme inhibitor to prevent plasma protein degradation.
4. The plasma samples were then stored at -70°C until analysis.

The plasma samples were thawed and then analyzed for urea nitrogen using the Berthelot method as described in section 3.2.2, except that 25µl of undiluted plasma or standard was added to the urease for incubation. The enzyme inhibitor added to plasma to prevent protein degradation was shown to have no effect on the assay by performing a direct comparison of samples with or without the inhibitor.

3.3 Determination of Total Nitrogen in Urine

The total nitrogen content of urine was measured using the Kjeldahl method devised by the Danish chemist Johann Kjeldahl (1849-1900) for estimating the nitrogen content of cereals (Fleck & Munro 1965). The substance for nitrogen analysis is digested by oxidation of the organic matter with sulphuric acid to reduce nitrogen to ammonium sulphate. This is then distilled with sodium hydroxide to liberate ammonia which is trapped in an acidic buffer with indicator and titrated with standardised sulphuric acid. The process was carried out using a Tecator Kjeltec semi-automated system (Perstorp Analytical, Bristol, U.K.)

1. To 1ml of urine or standard (3.3% $(NH_4)_2SO_4$ = 700mg Nitrogen) in a large distillation tube, 2 $CuSO_4$ Kjeltabs, 4 drops of antifoam and 12ml of concentrated sulphuric acid were added.
2. The distillation tubes were placed in a preheated block at 420°C for 10 minutes until the solution had been digested and turned green.
3. After leaving to cool, 70ml deionised water was added to each tube.
4. The digested sample was then steam distilled in a semi-automated system for three minutes after addition of 40% NaOH and the distillate collected into a conical flask containing 25ml boric acid indicator.
5. The dissolved ammonia solution was then titrated against 0.1M HCl until a colour change from blue to orange was observed. This allowed calculation of the nitrogen content of the urine sample.

3.4 Isolation of Urea from Urine

Urea was isolated from the urine samples using an ion-exchange resin in preparation for Isotope Ratio Mass Spectrometry (Jackson *et al* 1980).

1. A sample of urine containing approx. 3mg urea was brought to pH2 using cresol red as an indicator.
2. The resin (Dowex 50WX8-200 mesh H^+ form) was loaded onto a column to a height of 2cm and washed with deionised water.
3. The sample was loaded onto the column and washed through with 2ml deionised water when the last drop remained on the column.
4. The urea was then eluted from the column with 5ml volumes of disodium citrate buffer (21.01g citric acid and 8.0g sodium hydroxide made up to 1.0 litre at pH 3.41).
5. Before performing the assay on samples, the columns were validated to determine the volume of eluate which contained the eluted urea. A standard solution of 2mg urea-N was added to each column and eluted as above. A 25ul sample from each 5ml volume of buffer eluted was assayed for urea-N in triplicate using the Berthelot method (section 3.2.1). Results are shown in Table 3.1. Hence from this validation, volumes of 20-50ml were collected into a conical flask.
6. Using cresol red indicator the contents of the flask were adjusted to pH12 with 40% NaOH.
7. After adding anti-bumping granules, the eluate was reduced to 1ml on a hot plate and then immediately deep frozen at -20°C until ready for analysis.

Table 3.1 Validation of Elution Columns

COLUMN	ELUTION VOLUME (ML) CONTAINING UREA-N	% RECOVERY
1	30 - 50	79.05
2	30 - 50	85.41
3	30 - 45	82.15
4	25 - 45	82.40
5	20 - 40	97.85
6	20 - 35	89.50
7	20 - 35	96.70
8	25 - 35	88.04
9	20 - 35	87.55
10	20 - 30	97.30
11	25 - 40	103.50
12	20 - 35	105.30
13	20 - 35	105.40
14	20 - 40	110.95
15	25 - 45	95.60
16	25 - 35	92.25
17	25 - 40	92.95
18	20 - 35	84.50
19	20 - 35	90.60
20	20 - 35	99.10
21	30 - 45	88.65
22	20 - 40	86.70
23	30 - 45	96.65
	ie. collect 20 - 50 ml	

3.5 Liberation of N_2 Gas

The samples in which urea had been isolated from the urine and frozen at pH12 were treated in order to liberate the nitrogen gas for mass spectrometry.

1. The samples were transferred to clean acid washed glass tubes and frozen in a dry ice/propanol mixture.
2. Samples were evacuated in a low pressure system which allowed addition of 1 ml Lithium Hypobromite (4ml Bromine added to 120ml 10% LiOH on ice). This assumes that 1ml of the LiOBr solution is sufficient to liberate 5mg of N_2.
3. Test tubes were heated to react samples with the LiOBr solution and then refrozen. The liberated gas was collected through a liquid N_2 trap into gas containers.

3.6 Measurement of Isotopic Enrichment

The mass spectrometer measures the relative amounts of N_2 gas of different mass and allows assessment of the enrichment of the urea excreted in urine. Enrichment is a value which expresses the number of atoms of any particular isotope of an element in a sample as a fraction of the total number of atoms of that element present.

$$\text{ATOMS \%} = \frac{\text{number of 15-N atoms}}{\text{number of 15-N atoms+number of 14-N atoms}} \times 100$$

From the LiOBr reaction, N_2 gas is liberated from urea in a monomolecular reaction (Walser 1954). Hence, the relative proportions of $^{15}N^{15}N$ urea, $^{15}N^{14}N$ urea and $^{14}N^{14}N$ urea can be determined. Enrichment of the gas sample was measured in a triple collector isotope ratio mass spectrometer (SIRA 10, VG Isogas, Winsford, Cheshire, UK).

3.7 Inorganic Sulphate in Urine

Inorganic sulphate can be quantified by a method based on the turbimetry of sulphate as barium sulphate in the presence of a small amount of preformed barium sulphate. Polyethylene glycol (PEG) is used to stabilise the precipitate (Lundquist *et al* 1980).

1. A 0.02ml aliquot of sample or standard was diluted to 3.0ml with de-ionised water.
   Standards: 1.42g Na_2SO_4/100ml diluted to make standards of 0.5, 1.0, 1.5, 2.0, 2.5µmol/ml.
2. A 1ml aliquot of dilute HCL was then added followed by 1ml of Barium-PEG reagent.
   Barium-PEG reagent: 150g of PEG-6000 and 9.77g barium chloride dihydrate were made up to 1 litre in De-ionised water. A 0.2ml aliquot of 50mmol sodium sulphate (0.71g Na_2SO_4/100ml DIW) was then added to 100ml of the barium-PEG solution with efficient stirring. Stable for one week.
3. The solution was vortex mixed and left to stand for 5 minutes before determining the absorbance at 600nm with a spectrophotometer.

3.8 5-Oxoproline in Urine

The analysis of this marker of glycine status in the individuals studied was carried out by Dr. C. Persaud using the method described in his PhD Thesis (Persaud 1995). In this assay, 5-oxoproline isolated from free glutamate is first converted to glutamate by acid hydrolysis and then measured enzymatically with glutamate dehydrogenase.

1. 2ml of acidified urine or standard was applied to a small column containing Dowex 2 (chloride form) resin. The column was eluted with 3ml of 0.2M HCl, and the eluate applied to the top of a second column, filled with Dowex 50 (hydrogen form) resin. The second column was eluted with 3ml deionised water.

2. To this eluate, 3ml of 6M HCl was added and the solution placed in a water bath at 100°C for 2 hours. The solution was evaporated, and the dried residue resuspended in 2ml of Tris-hydrazine buffer pH9. The solution was mixed and filtered. This is referred to as the 'test' solution.

   Tris-hydrazine buffer - 4ml 2M Tris + 4ml Hydrazine + 2ml 0.1M EDTA/Na dissolved in 60ml deionised H_2O, pH adjusted to 9 using 1M HCl and final volume made up to 80ml with deionised H_2O.

3. The glutamate content was measured using glutamate dehydrogenase and the increase of NADH, as measured by the absorbance at 340nm, being proportional to the amount of L-glutamate converted to 2 oxoglutarate.For the assay, 200ul of the test solution was added to 800ul of the common medium.

   Common Medium - 0.5ml Tris-hydrazine buffer + 3.3µl 0.1M ADP + 3.3µl 2% NAD + 6.7µl GDH in glycerol (10mg protein/ml, Boeringer Mannhein) + 3ml H_2O.

4. Tubes were incubated at 37°C for 30 minutes and then read at 340nm in a spectrophotometer. Each solution was read against its own blank containing the reagents and solution without the enzyme. A standard curve was plotted which allowed estimation of the 5-oxoproline content of the urine samples.

3.9 BMR and Anthropometry

Anthropometrical measurements were taken by Mrs. Sarah Bond. Height, weight, mid arm circumference and skinfold measurements were recorded. From the four skinfold readings, taken with Harpenden calipers, the lean body mass can be calculated for both men and women using the equations of Durnin & Rahaman (1967).

Equations:-

$$\% \text{ Body Fat} = [\ 4.95/D - 4.5\ ] \times 100$$

where $D = 1.1610 - 0.0632\ (\times \log^{10} \text{sum of skinfolds})$ for men

and $D = 1.1581 - 0.0720\ (\times \log^{10} \text{sum of skinfolds})$ for women

Basal metabolic rates were measured by indirect calorimetry (Datex Deltatrac) at an average time of 07.30 hours and after a 12 hour fast. Measurements of VCO_2 and VO_2 were taken over a 30 minute period after a five minute rest period to allow the subject to become relaxed in the hood. From these readings RQ and resting energy expenditure were derived.

3.10 Statistical Analysis

All results are expressed as mean $\pm$ SEM. Statistical analysis was carried out using the ARCUS professional II software. Paired t tests were used to determine if differences between studies were statistically significant. However, because of the small groups sizes, non-parametric statistics, in the form of the Mann Whitney U or Wilcoxon Signed Ranks test for paired analysis were also performed.

CHAPTER 4

DIURNAL CYCLING IN UREA-NITROGEN HYDROLYSIS

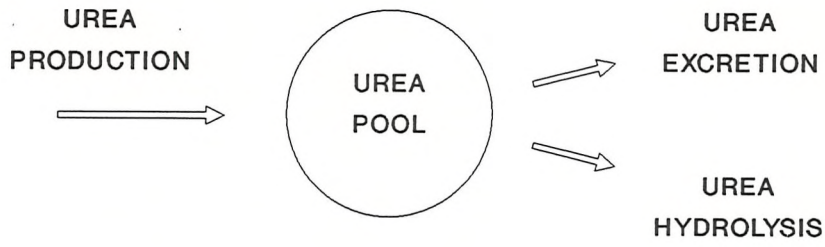
4.1 Introduction

Despite homeostasis, many components of the internal environment can vary within a small range depending on the time of day. Protein metabolism can be affected by hormones, food intake, and other factors which display diurnal rhythmicity. However, the most fundamental influence on the diurnal cycle is nutritional. That the periodic nature of feeding and fasting results in changes in body protein and nitrogen balance has long been recognised. It has been demonstrated in rats (Garlick *et al* 1973, Millward *et al* 1974) and man (Garlick *et al* 1980, Clugston & Garlick 1982, Rennie *et al* 1982), that protein turnover alters between the fed and fasted states. These studies illustrate the bodies adaptive mechanism to fasting in that, during the fed state protein synthesis increases relative to fasting in order to store the rise in plasma amino acids. Recent studies have demonstrated that on feeding, insulin inhibits degradation and amino acids both stimulate synthesis and oxidation and also further inhibit degradation (Pacy *et al* 1994). It has been suggested that the fasting losses of protein degradation creates the metabolic demand for protein and could reflect dietary requirements (Millward & Rivers 1988).

So how do these changes affect the three major components of the urea kinetics model (Figure 4.1)? The daily fluctuations in protein metabolism are reflected by altering nitrogen losses from the body. A diurnal cycle in the excretion of urinary urea has been observed (Steffee *et al* 1981, Parsons *et al* 1983).

In two recent comprehensive studies, diurnal alterations in nitrogen metabolism were measured using isotopically labelled amino acids (Price *et al* 1994, Quevedo *et al* 1994) and the amplitude of the change found to be primarily influenced by protein intake.

Figure 4.1 A Simplified Model of Urea Kinetics



However, these studies did not measure urea production directly. Data published from studies by El-Khoury *et al* (1994a,1994b) further demonstrated diurnal cycling in leucine metabolism and went on to describe changes in urea kinetics as measured by $^{15}N^{15}N$ urea. These results were recalculated by Jackson (1994b) to illustrate that although urea production remained constant throughout a 24 hour period, urea excretion, and hence hydrolysis, showed a diurnal variation with hydrolysis being minimal during the day and rising to 50% of production during the overnight fast.

Hence, the aim of the present study was to directly assess the diurnal fluctuations in urea kinetics using the stable isotope $^{15}N^{15}N$ urea.

4.2 Protocol and Methods

4.2.1 Protocol

The study was carried out in seven normal adult men aged 21-23 years. The subjects agreed to take part after the nature of the investigation was explained. They were all in good health at the time of the study. The study had the approval of the Southampton Hospitals and South West Hampshire Authority Ethical Subcommittee.

The protocol lasted for five days over which all food was provided in order to supply a controlled intake of 70g protein/day. Each subject had his basal metabolic rate measured along with other anthropometrical data. Complete collections of urine were collected at three hourly intervals over the final 27 hours and urea kinetics were calculated over this period.

4.2.2 The 70g Protein/day Diet

The 70g protein/day diet was a reflection of an adequate daily intake for adult males in the UK (Gregory *et al* 1990). Habitual food was used rather than a liquid formula intake, despite a lessened sense of accuracy. The diet shown in Table 4.1 was designed by weight using food composition tables (McCance & Widdowson 1991). The diets were designed to provide sufficient energy using the calculation of BMR x 1.4 as advised by the DRV energy values for a sedentary lifestyle (HMSO 1991). On the fifth day, feeding took place at three hourly intervals from 06.00 to 21.00 in as near to isonitrogenous amounts as could be achieved with whole foodstuffs.

Table 4.1 The 70g Protein/day Diet

	PORTION SIZE	PROTEIN CONTENT (g)	ENERGY CONTENT (kJ)
BRANFLAKES	45g	4.6	605
WHOLE MILK	265ml	8.5	735
ORANGE JUICE	150ml	0.6	210
APPLE	2	0.4	353
BANANA	1	0.9	265
CHEESE\HAM SANDWICH	3 sl	17.9	1999
HULA HOOPS	35g	1.2	218
JAFFA CAKES	4	1.4	613
CHOCOLATE DIGESTIVES	4	4.0	1243
MEALS:- CHOICE OF			
	300g	22.1	1168
1. CHICKEN TIKKA MASALA	60g	4.3	1210
BROWN RICE	135g	3.3	1222
CHOCOLATE DESSERT			
	300g	20.4	1604
2. LASAGNE	150g	1.9	437
SALAD	125g	4.0	794
YOGHURT			
	300g	24.9	903
3. CHICKEN DO PIAZA	40g	4.7	1029
SPAGHETTI	145g	1.3	760
PEACHES AND CREAM			
TOTAL		68.5	9841

4.2.3 Urine Collection

A baseline urine sample was collected from 21.00 to 00.00 on day 4 before the prime dose of isotope was administered. Three hourly collections began at 06.00 on the fifth day until 09.00 the following morning. Urine was collected into 500ml plastic containers containing 2ml 6M HCl/100ml as a preservative.

Volume was measured and then, in order to store urine until analysis, a 60ml alloquot was placed into plastic containers and frozen at -20°C. Remaining urine was disposed of and the plastic containers washed with bleach to allow them to be reused.

4.2.4 Urea kinetics

The protocol used a prime intermittent dose method of urea kinetics. A known amount of isotope ($^{15}N^{15}N$ urea, 98% atoms ^{15}N, Cambridge Isotope Laboratories, Cambridge, MA, U.S.A.), ie. about 94.5mg was made up in 36ml of sterile water and then divided into a prime dose of 10ml (containing approx. 27.5mg) and the remaining for 2ml intermittent doses (containing approx. 5.5mg). This was stored at 4°C until use.

The prime dose was taken orally at midnight on day 5 in order reduce time taken to reach a steady state plateau in urinary urea enrichment. From 06.00 hours, intermittent doses of urea isotope were administered at 3 hourly intervals. At these three hourly intervals, urine was collected prior to dose administration. Food was consumed between 06.00 and 21.00 only, in order to maintain the normal fasted state overnight. This protocol was continued until 09.00 the next day, ie. a 27 hour period.

4.2.5 Analysis

Each urine sample was analysed for ammonia and urea nitrogen using the Berthelot method (section 3.2). Urine was extracted from the urine using ion-exchange columns (section 3.3) and the nitrogen gas liberated from these samples (section 3.4) analysed in the mass spectrometer for relative intensities of urea isotopes (section 3.5).

4.3 Results

4.3.1 Anthropometry

All studies were completed satisfactorily and diets were consumed as planned. Anthropometrical measurements from the subjects are shown in Tables 4.2 to 4.4. The energy intake of 126kJ/kg/d was adequate to a level of BMR x1.4 and maintained body weight in all subjects.

Table 4.2 Measured Anthropometrical Data

	AGE Yrs	WT kg	HT cm	BI mm	TRI mm	SUB SCAP mm	SUP RAIL mm	MAC mm
1	21	72.2	191.5	5.4	10.5	8.5	3.0	255
2	21	71.1	179.5	5.0	5.1	8.5	3.9	285
3	22	73.3	173.4	11.8	11.6	24.0	7.1	300
4	22	84.6	181.8	11.4	9.3	10.3	3.7	305
5	22	71.4	183.3	14.3	12.5	12.3	5.4	285
6	22	80.4	178.2	10.5	11.8	12.5	5.2	330
7	23	63.6	168.4	11.2	11.8	12.1	6.0	275

WT = Weight
HT = Height

Skinfolds:
BI = biceps
TRI = triceps
SUBSCAP = subscapular
SUPRAIL = suprailiac
MAC = mid arm circumference

From these results % body fat and hence lean body mass can be calculated using the equations of Durnin (section 3.9). Also body mass index (Wt/Ht^2) is stated in Table 4.3.

Table 4.3 Calculated Anthropometrical Data

	BODY MASS INDEX	% BODY FAT	TOTAL BODY FAT (kg)	LEAN BODY MASS (kg)
1	19.6	12.6	9.1	63.1
2	21.9	10.1	7.2	63.9
3	24.4	21.0	15.4	57.9
4	25.5	15.4	13.0	71.6
5	21.3	18.4	13.1	58.3
6	25.4	17.1	13.7	63.3
7	22.4	17.4	11.1	52.5

The subjects basal metabolic rate was measured using indirect calorimetry.

Table 4.4 Basal Metabolic Rate Data

	$\dot{V}O_2$ ml/min	$\dot{V}CO_2$ ml/min	RQ	REE kJ/day	E REQ kJ/day
1	263	233	0.89	7660	10724
2	249	222	0.89	7260	10164
3	233	262	0.89	7650	10710
4	259	215	0.83	7440	10416
5	251	227	0.90	7340	10276
6	244	214	0.88	7070	9898
7	201	175	0.87	5800	8120

REE = resting energy expenditure
E REQ = energy requirement (REE x 1.4)

4.3.2 Urea Kinetics - Production

The ratio of $^{15}N^{15}N$ urea/$^{14}N^{14}N$ urea (30/28 urea) was measured in the three hourly urine samples using isotope ratio mass spectrometry. The calculations used to derive urea production from these ratios are shown below in Table 4.5 using Subject 1 as the example.

Table 4.5 Calculation of Urea Production Rates from urinary enrichment of 30/28 urea in Subject 1 over a 27 hour period.

δI30	It	δ30 mmol ^{30}N/h	Pu mmol/h	Pu mg/kg/h
0.17587	100.218	0.0572	32.595	6.320
0.18869	100.237	0.0572	30.886	5.892
0.18359	100.236	0.0572	31.230	6.056
0.14363	100.180	0.0572	39.896	7.736
0.15761	100.200	0.0572	36.365	7.051
0.16678	100.233	0.0572	34.377	6.666
0.13163	100.174	0.0572	43.532	8.441
0.16612	100.217	0.0572	34.508	6.691
0.12882	100.177	0.0572	44.482	8.625
0.11267	100.152	0.0572	46.700	9.055

where δI30 = enrichment of 30/28 urea minus the baseline enrichment

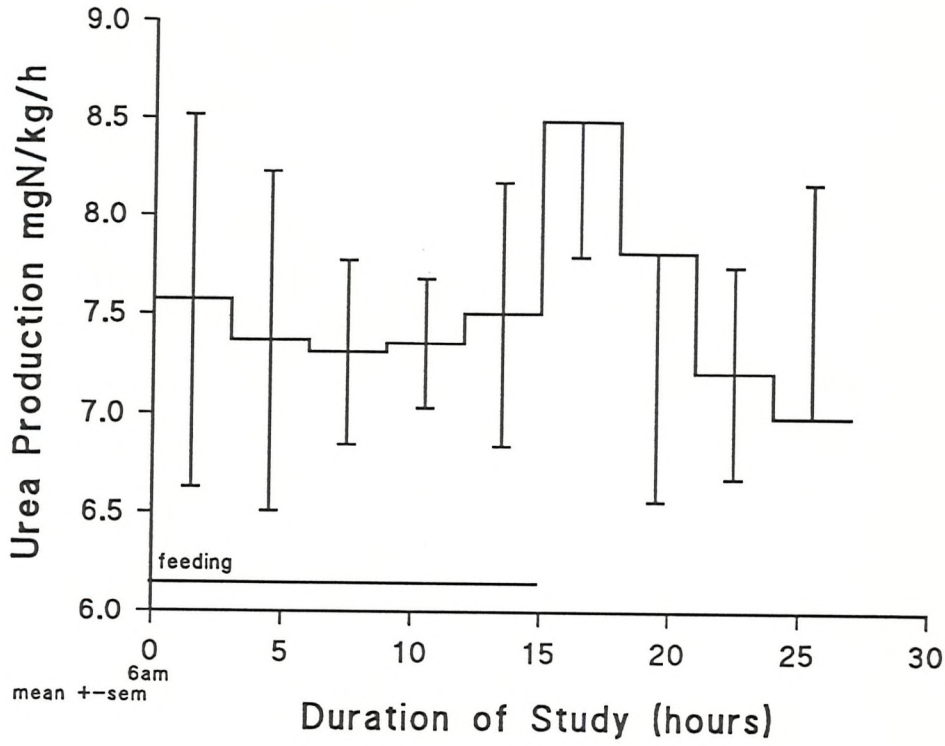
It = 100 + enrichment of 30/28 + enrichment of 29/30

δ30 = dose of $^{15}N^{15}N$ urea administered as label, mmol ^{30}N/h

$$\text{Urea Production} = \delta 30 \times \frac{It}{\delta I30} \text{ mmol N/h}$$

Over the 24 hour period, urea production in this individual ranged from 5.9 to 9.1 mg/kg/h. The same process was applied to the urinary enrichment values for the other six subjects (Appendix 2a), the mean production rate for each three hour period calculated and shown in Figure 4.2. None of the three hourly periods were significantly different from any other and hence, despite individual variations, mean production rate is constant throughout a 24 hour cycle of feeding and fasting.

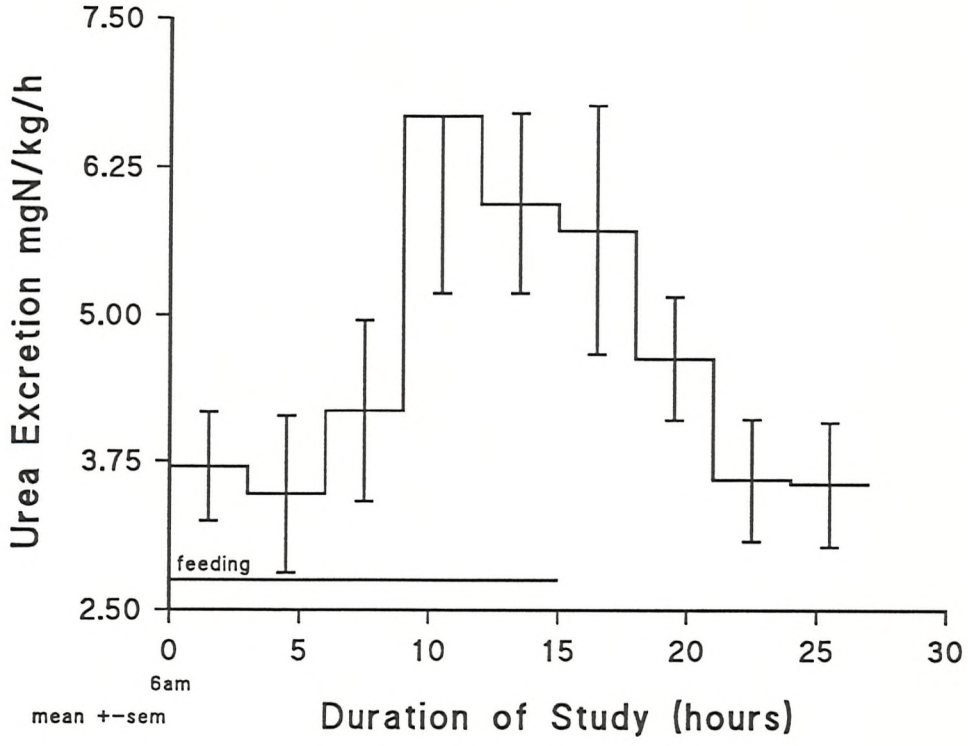
Figure 4.2 Mean urea production rates over a 27 hour period for seven adult males fed 70g protein/day.



4.3.3 Urea Kinetics - Excretion

Urea excretion rates were assessed by direct analysis of urinary urea content (see methods section 3.3) in each three hourly collection for each subject (Appendix 2b). Mean urea excretion values for the 24 hour period are shown in Figure 4.3. There is a significant rise in excretion during the afternoon, peaking between 3pm and 6pm. Excretion then falls so that lowest levels are overnight. When compared to the mean value for the entire 24 hour period (4.71 mgN/kg/h) excretion was consistently above average from 1500-2400h and consistently below average between 0300-1200h.

Figure 4.3 Mean urea excretion rates over a 27 hour period for seven adult males fed 70g protein/day.



4.3.4 Urea Kinetics - Hydrolysis

The difference between urea production and that excreted is attributed to the proportion of urea production which passes to the colon and undergoes hydrolysis. Hence, hydrolysis mirrors the changes in urea excretion. This is shown diagrammatically in Figure 4.4. Six to nine hours after feeding, the hydrolysis of urea-nitrogen decreased and was on average 26% of urea production. Six hours after feeding had ceased, salvage increased to an average of 50% of urea production. From these data, it can be seen that urea

hydrolysis is minimal during the day, but during the overnight fast, 50% of urea production is hydrolysed in the colon.

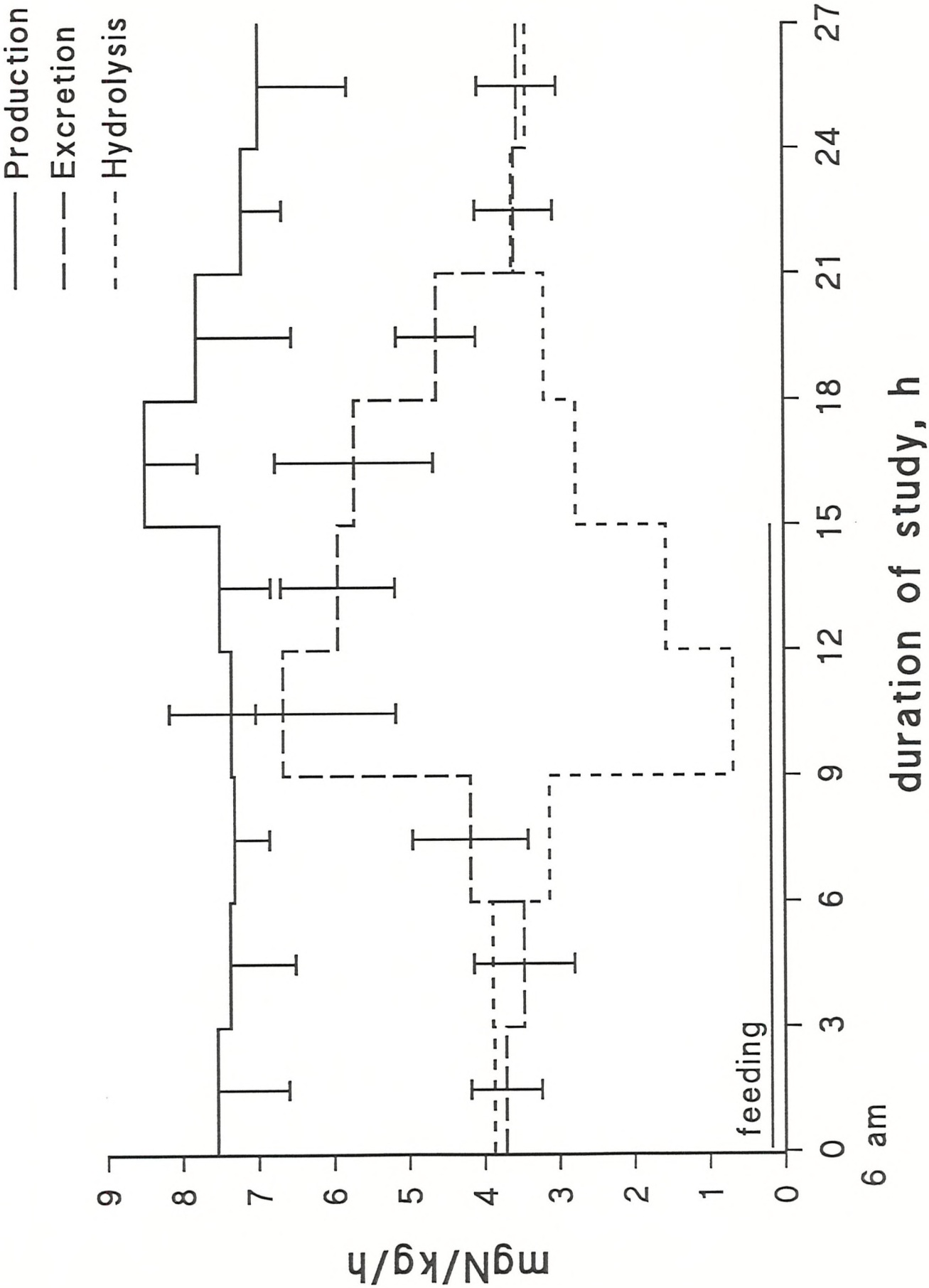
Looking at these results in a different way, 12 hourly periods were compared for statistical difference and also urea kinetics for these periods compared to answers over a 24 hour study period (Table 4.6).

Table 4.6 Urea kinetics calculated during different time periods for seven adult males fed 70g protein/day.

TIME OVER WHICH UREA KINETICS WERE CALCULATED	PRODUCTION mgN/kg/h	EXCRETION mgN/kg/h	HYDROLYSIS mgN/kg/h
24 HOURS	7.65 ±0.3	4.71 ±0.3	2.94 ±0.4
3AM-3PM	7.51 ±0.5	3.70 ±0.3	3.81 ±0.5
3PM-3AM	7.80 ±0.4	5.74 ±0.5*	2.06 ±0.6*
6AM-6PM	7.54 ±0.5	4.64 ±0.5	2.90 ±0.5
6PM-6AM	7.77 ±0.4	4.97 ±0.4	2.80 ±0.4
9AM-9PM	7.67 ±0.4	5.26 ±0.5	2.41 ±0.4
9PM-9AM	7.64 ±0.5	4.37 ±0.4	3.27 ±0.5
12AM-12PM	7.36 ±0.5	3.92 ±0.3	3.44 ±0.5
12PM-12AM	7.97 ±0.4	5.63 ±0.6*	2.34 ±0.4*

* mean is significantly different between the two 12 hour periods, $P<0.05$.

Figure 4.4 Urea Kinetics over a 27 hour period for seven adult males fed 70g protein/day.



From these data, it can clearly be seen that urea-nitrogen hydrolysis is significantly higher between 12am-12pm compared to 12pm-12am and also increased between 3am-3pm when compared to 3pm-3am. None of the values reached over 12 hourly periods were significantly different to the average value for a 24 hour period. However, values gained for urea kinetics appear to be dependent of the time period over which the measurements are taken.

4.4 Discussion

The purpose of this study was to examine by direct measurement the diurnal alterations in urea-nitrogen salvage, previously only calculated from ^{13}C leucine studies (Jackson 1994 from El-khouri *et al* 1994). The literature describes differences in protein turnover and urea excretion between day and night, thought to be due mainly to the absence of food intake over night. Hence, it is logical that along with these changes in nitrogen metabolism, the process of nitrogen recycling in the colon by the colonic microflora would also follow the same trends.

The experimental design was essentially the standard protocol used in our laboratory. The primed intermittent infusion was given three hourly as the subjects were free living and the method is designed to be non-invasive. Although hourly measurements might have improved the interpretation of results, this would have seriously encroached on the individuals ability to carry out their daily tasks. Subjects went about their daily work and were not restricted to a metabolic unit. This is also why food and urine was collected three hourly and why plasma samples were not taken throughout the study.

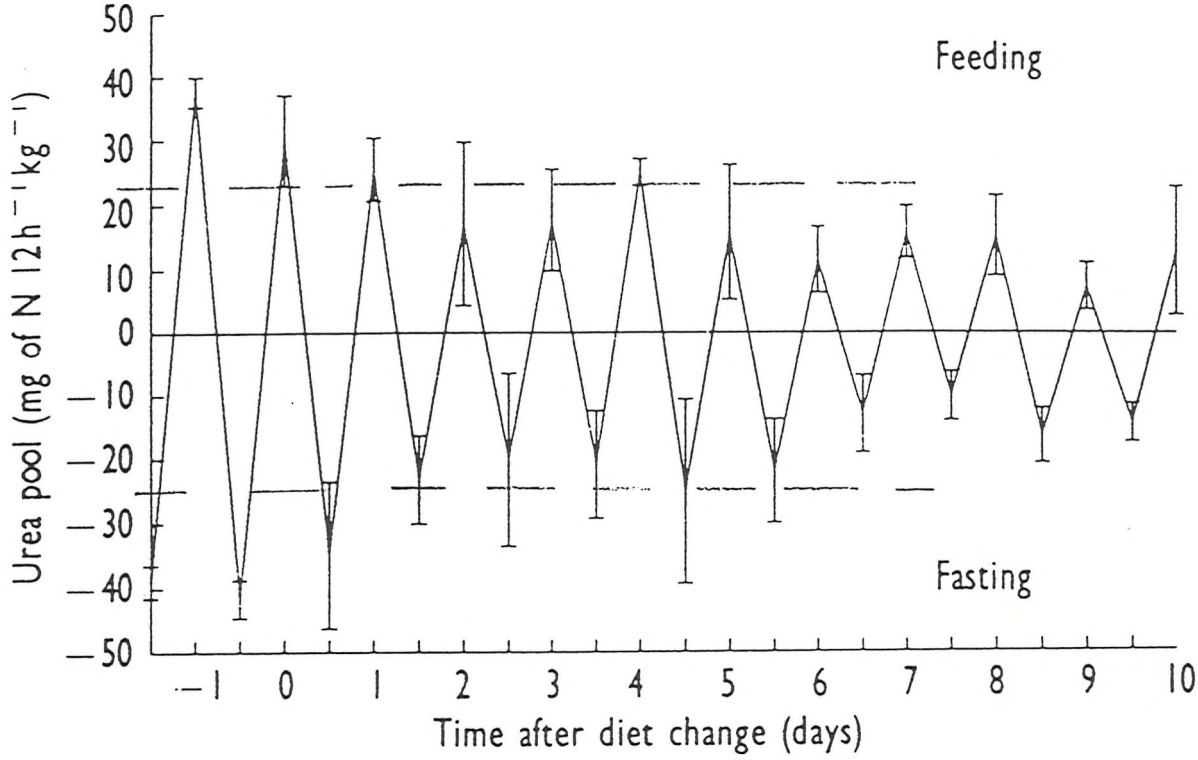
Urea production was measured directly as the ratio of doubly labelled isotope $^{15}N^{15}N$ urea to unlabelled $^{14}N^{14}N$ urea in the urine. Despite a daily cycle of food intake and over night fast, urea production remained remarkably constant with the maximum variation from the mean value only 10%. This implies that amino acid oxidation was constant over this period. Previously, studies measuring amino acid oxidation directly using ^{13}C or ^{14}C leucine have demonstrated an increase in oxidation on during the day whilst receiving a protein intake at similar levels or higher than the 70g protein/day given here (Garlick *et al* 1980, Motil *et al* 1981a, Hoffer *et al* 1985, Pacy *et al* 1994, El-Khouri *et al* 1994a). At levels of protein intake lower than this, no increase in oxidation was demonstrated on

feeding in some studies (Motil *et al* 1981, Yang *et al* 1986) and an increase demonstrated in others (Price *et al* 1994). The 70g protein/day intake may be at the margins of the change in response and measuring urea production may not indicate any changes in amino acid oxidation.

Urinary urea excretion was higher during the day, rising after midday and falling during the evening, in agreement with other studies (Steffee *et al* 1981, Parson *et al* 1983, Grove 1993) and the same as results shown for total urinary nitrogen excretion (Price *et al* 1994, Quevedo *et al* 1994). Garlick was unable to demonstrate changes in excretion which reflected alterations in protein oxidation and attributed this to the large size of the body urea pool which 'smoothed out' fluctuations in the rate of urea excretion (Garlick *et al* 1980). If urea production is constant and excretion falls, the urea must either pass to the colon for hydrolysis or is retained in the body pool. Hence, the importance of urea pool size should be considered.

The model of urea kinetics assumes a steady state during administration of isotope ie. that the input and outputs to the urea pool are constant and therefore pool size is constant. This ensures that the ratio of urea in the pool to the dose of label entering the pool are consistent with the ratio of urea to dose in the pool and leaving the pool ie. the point of measurement. However, it has been demonstrated that the urea pool expands during protein intake and contracts on fasting (Fern *et al* 1981, Price *et al* 1994, Quevedo *et al* 1994). The magnitude of this expansion is related to protein intake. In this study, plasma urea was not measured on a three hourly basis and so the changes in the size of the urea pool over the 24 hour period is unknown. However, from the data given by Quevedo *et al* from figure 1 (page 188 of this reference) shown in Figure 4.5, we estimate that on a 70g protein/day intake, the extent of this variation is likely to be around $\pm$2mg/kg/d.

Figure 4.5 Diurnal changes in plasma urea concentration in subjects changing from a 135g/day to 58g/day protein intake (from Quevedo 1994). Estimation of diurnal variation on 70g/day protein taken to be 24mgN/kg/12 hours.



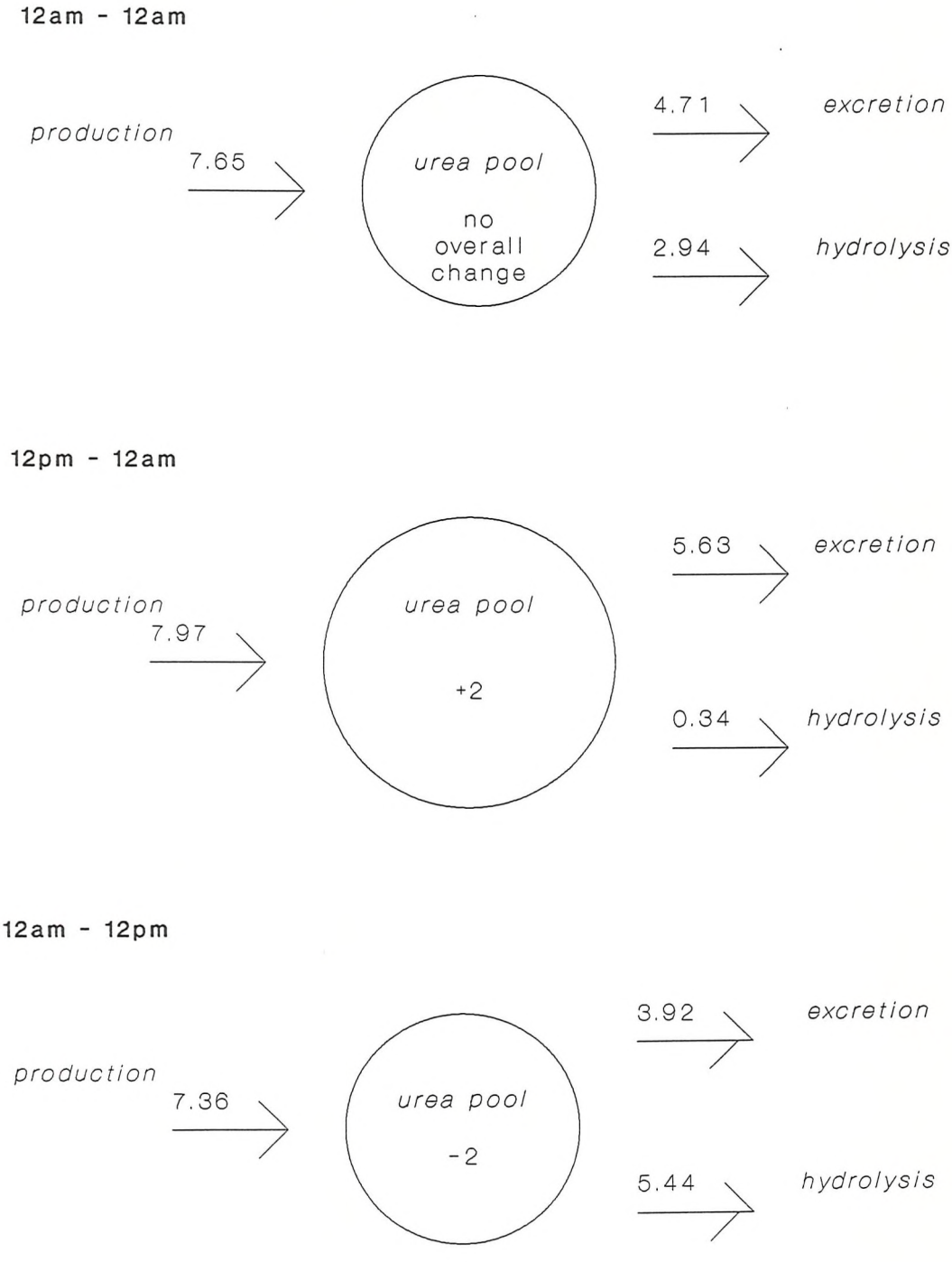
Measuring urea kinetics over a 24 hour period would alleviate the need to correct for alterations in pool size.

Many investigators now routinely correct measured excretion rates for changes in the urea pool when estimating urea production. Alterations in pool size will influence the amount of urea production which is excreted from the pool and measured in the urine. When the size of the urea pool increases during feeding, urinary urea will underestimate production as some of the urea produced is retained in the pool. Conversely, as the pool contracts urea is lost from the pool as well as that

produced from the urea cycle and hence, excretion will be an overestimate of production. Changes in pool size will not affect the measurement of urea production using $^{15}N^{15}N$ urea as this allows direct measurement of production from the ratio of urea to dose entering the pool.

Using the model shown in Figure 4.1, if urea production is measured directly and excretion is measured in the urine, alterations of pool size may only influence the estimation of the rate of urea hydrolysis. If the pool is expanding during the day, then the difference between urea production and that excreted, which is attributed to have been hydrolysed in the colon (T=Pu-Eu), could have, in fact, been retained in the pool. In this study, it would have been useful to have the plasma urea data to enable correction of hydrolysis for expansion of the urea pool. However, if the value of $\pm$2mg/kg/d is taken to estimate changes in pool size, the results shown in Figure 4.4 will be altered in the following manner (Figure 4.6). The effect is to accentuate the diurnal changes in urea hydrolysis.

Figure 4.6 Diurnal changes in urea kinetics over two time periods corrected for changes in urea pool size estimated to be ±2mgN/kg/h (units expressed as mgN/kg/h).



As the proportion of urea production which is not accounted for by urea excretion is assumed to have passed to the colon, with corrections for changes in pool size, urea-nitrogen hydrolysis mirrors the diurnal changes in rates of excretion. These observations are in agreement with those predicted from the work of El-Khouri (Jackson 1994 from El-khouri 1994). Hydrolysis and hence, salvage of urea-nitrogen varies between day and night. Hydrolysis is minimal during the day and rises to 50% of production overnight. The decrease in the rate of urea-nitrogen hydrolysis can be seen to occur approximately 6 hours after feeding commenced. As small intestinal transit time is around 6 hours, these results could support the suggestion that the rate of salvage is determined by nitrogen substrate available to the bacteria. Hence, nitrogen intake could be a primary influence on the salvage system on a diurnal basis. One way of resolving this theory would be to carry out a further study in which feeding and fasting periods were varied throughout the 24 hour period ie. fasting during the habitual daylight hours.

From this data, it can be confidently concluded that urea production rates are not reflected in the excretion of urinary urea. With these observed changes in urea excretion and hence, hydrolysis, care must be taken when using urea excretion to calculate other parameters in nitrogen metabolism. If measurements are not taken over a 24 hour period, results are likely to be affected by the time period over which measurements are taken due to the dampening effect of urea pool size changes, and by the intensity and timing of nitrogen intake.

4.5 Summary

The conclusions drawn by Langran *et al (1992)* and Danielsen & Jackson (1992) considered the hydrolysis and consequent salvage of urea-nitrogen to be a fundamental aspect of metabolic adaptation to a lowered nitrogen intake over a period of days. The present study demonstrates that this system could also be of significance in enabling the body to sustain homeostasis in the short term, in response to a diurnal cycle of food intake. During the overnight fast, urea salvage appears to increase to compensate for a deficit in nitrogen intake. It is demonstrated that urea excretion rates do not reflect endogenous production and hence, there are clear implications for the calculation of non-protein respiratory quotient by indirect calorimetry and in the derivation of protein degradation in studies of protein turnover.

CHAPTER 5

THE INFLUENCE OF NON-ESSENTIAL NITROGEN ON COLONIC SALVAGE

5.1 Introduction

The factors which exert an influence on, or control, the urea salvage mechanism are of greatest importance if an understanding of nitrogen metabolism is to be reached. Previous studies have demonstrated an adaptive role for urea salvage determined by the magnitude of protein intake. On lowering intake to below 70g/day in men, urea salvage increases in order to recover the nitrogen deficit until a minimum dietary requirement of 35g protein/day is reached (Langran *et al* 1992, Danielsen & Jackson 1992). It has become increasingly recognised that estimation of protein requirement should be considered in terms of total nitrogen. Hence, determining the influence of nitrogen intake on urea salvage is fundamental.

Nitrogen intake can be visualised as comprising of two nitrogen sources; essential amino acids and non-essential nitrogen. The view that only essential amino acids are limiting factors in the diet requires re-evaluation if studies looking at non-essential nitrogen are examined. In attempting to define the minimal requirements of essential amino acids for humans, both for adults and children, investigators came to the conclusion that a source of nitrogen for body synthesis of the non-essential amino acids was needed and maintenance requirements were influenced by the level of total nitrogen in the diet.

Early studies by Rose demonstrated in rats that with a minimum essential amino acid intake, the limiting factor is the inability of cells to synthesize non-essential amino acids due to a shortage of total nitrogen, but this is relieved by supplementing alternative nitrogenous compounds (Rose 1949, Rose & Wixom 1955). Swendseid discovered that nitrogen balance was achieved when essential amino acids accounted for only 6.5-8.5 per cent of total nitrogen in women and 10-13 per cent in

men (Swenseid *et al* 1962, 1961). In a fundamental study, infants were fed decreasing quantities of milk protein in order to decide which was the most limiting amino acid of the diet and it was found that weight gain and nitrogen retention could be restored to normal by supplementing non-essential nitrogen in the form of urea or glycine (Snyderman *et al* 1962). Thus non-essential nitrogen appeared to be the most limiting factor.

There is a substantial body of evidence which shows that the dietary requirements for essential amino acids can be reduced if the total nitrogen content of the diet is increased. Numerous studies by Kies using the nitrogen balance technique have demonstrated this effect and suggest the possibility of urea as a non-essential nitrogen source for humans (Kies *et al* 1972a, 1978). Nitrogen retention in men was improved when total dietary nitrogen was increased but essential amino acid intake kept constant (Kies 1975). This response was also seen when a corn diet in which the first limiting factor was non-essential nitrogen (Kies *et al* 1963) was supplemented with non-essential nitrogen (Kies *et al* 1965a, 1972b, Korsland *et al* 1977). However, the nature of this response to non-essential nitrogen has never been adequately explained and no studies carried out to determine whether this is due to a stimulation of urea hydrolysis and salvage.

However, there is a further question underlying the use of exogenous urea supplementation. It has been argued that any functional relevance of urea hydrolysis can only be adequately demonstrated if there is a deterioration of nitrogen balance and function when urease activity is inhibited by the use of antibiotics (Walser 1981). In one study where this approach was adopted, there was not a dramatic response to antibiotics and this has been used as the basis for rejecting any functional relevance of the system (Mitch *et al* 1977, Mitch & Walser 1977). However, this study was carried out in patients with severe renal failure in whom the intake of nitrogen as a mixture which included essential amino acids was between 24-26g protein, below the level where the salvage system operates

effectively. In another study, dietary urea supplements improved nitrogen balance in malnourished adults, an effect which was reduced by treatment with neomycin (Tripathy *et al* 1970). Thus, the alternative approach would be to take individuals in whom protein intake is too low to sustain nitrogen balance, and to see whether balance is achieved with the addition of dietary supplements of urea.

The objective of the present study was to address these questions directly by exploring the effect of dietary urea supplementation on the urea salvage system in subjects in whom the dietary protein intake was inadequate to maintain nitrogen balance.

5.2 Protocol and Methods

5.2.1 Protocol

The studies were carried out in six normal adult men aged 21-23 years. The subjects agreed to participate after the nature of the study had been explained to them. They were all in good health at the time of the studies. The study had the approval of the Southampton Hospitals and South West Hampshire Health Authority Ethical Subcommittee. Each subject underwent four protocols (Table 5.1), each of which lasted five days. For each of the protocols, the subject was provided with a standard diet to eat over the five day period.

Table 5.1 The Dietary Protocol

	PROTEIN	UREA	TOTAL NITROGEN
REFERENCE	70g	---	11.2g
LOW PROTEIN	30g	---	4.8g
SUPPLEMENT 1	30g	6.9g	8.0g
SUPPLEMENT 2	30g	13.7g	11.2g

Each subject had his basal metabolic rate measured (Indirect calorimetry using Datex Deltatrac) along with other anthropometric data. Percentage body fat and lean body mass were calculated from skinfold measurements using the equations of Durnin (see section 3.9). Complete collections of urine were made for the duration of the study, and during the last 24 hours of each study period measurements of urea kinetics were made. A plasma sample was taken at 3pm on day five and stored at -70°C. The studies were carried out in the order shown and at least three weeks passed between consecutive studies.

5.2.2 Diets

Each subject consumed the diets listed (Tables 5.2 and 5.3). Two diets were compiled with 70g protein/day intake (the reference diet, REF) which was considered to be adequate and a 30g protein/day intake (low protein diet, LP) which was considered to be marginally inadequate (Danielsen 1992). On two occasions the low protein diet was supplemented with urea, at either 6.9g/day (LP-U1) or 11.2g/day (LP-U2). The higher supplement brought the total nitrogen intake to a level which was the same as the REF diet, whilst the lower supplement gave a total nitrogen intake which was low, but would have been adequate had the total nitrogen been provided as protein, 50g/day (Langran 1992).

Despite a lessened sense of accuracy in protein intakes, it was decided to use habitual food rather than a liquid diet intake. For each diet three different menus were designed by weight using food composition tables (McCance & Widdowson 1991) for variety over the five day study period, and then checked using the Comp-eat computer analysis to enable micronutrients and amino acids to be examined (Appendix 1). Food questionnaires were answered by each subject to determine personal preferences. The two dietary intake menus were as similar as possible, ie the same meals simply altered to a vegetarian version to lower protein intake. It has been shown that differences in urea kinetics between vegetarians and omnivores are not likely to be due to protein quality, but total protein intake (Bundy 1993). The diets were designed to provide sufficient energy using the calculation of BMR x 1.4 as advised by the DRV energy values for a sedentary lifestyle (HMSO 1991). The supplements of urea in studies 3 and 4 were given as a flavoured drink at three intervals during the day ie. with meals.

Table 5.2 The 70g Protein/day Diet

	PORTION SIZE	PROTEIN CONTENT (g)	ENERGY CONTENT (kJ)
BRANFLAKES	45g	4.6	605
MILK	265ml	8.5	735
ORANGE JUICE	150ml	0.6	210
APPLE	2	0.4	353
BANANA	1	0.9	265
CHEESE/HAM SANDWICH	3 sl	17.9	1999
HULA HOOPS	35g	1.2	218
JAFFA CAKES	4	1.4	613
CHOCOLATE DIGESTIVES	4	4.0	1243
MEALS:- CHOICE OF			
1. CHICKEN TIKKA MASALA	300g	22.1	1168
BROWN RICE	60g	4.3	1210
CHOCOLATE DESSERT	135g	3.3	1222
2. LASAGNE	300g	20.4	1604
SALAD	150g	1.9	437
YOGHURT	125g	4.0	794
3. CHICKEN DO PIAZA	300g	24.9	903
SPAGHETTI	40g	4.7	1029
PEACHES AND CREAM	145g	1.3	760
TOTAL		68.5	9841

Table 5.3 The 30g Protein/day Diet

	PORTION SIZE	PROTEIN CONTENT (g)	ENERGY CONTENT (kJ)
RICE KRISPIES	30g	1.8	466
MILK	80ml	2.5	223
ORANGE JUICE	100ml	0.4	139
JAM SANDWICH	2 sl	5.5	1478
HULA HOOPS	35g	1.2	218
APPLE	1	0.2	176
BANANA	1	0.9	265
JAFFA CAKES	2	0.7	307
BOILED SWEETS	65g	---	895
CREAM	100g	1.6	1882
LUCOZADE	250ml	---	714
MEALS:- CHOICE OF			
1. VEGETABLE CURRY/RICE	300g	7.2	1231
PASTA/SWEETCORN SALAD	140g	5.3	1453
PEACHES AND CREAM	145g	1.0	819
2. VEGETABLE LASAGNE	300g	8.4	1012
SALAD AND COLESLAW	200g	2.0	374
YOGHURT	125g	4.0	424
3. MUSHROOM DO PIAZA	300g	9.0	1218
SPAGHETTI	40g	4.0	655
SALAD AND COLESLAW	200g	2.0	374
PEARS AND CREAM	145g	1.0	819
TOTAL		29.7	9829

5.2.3 Urea Kinetics

The protocol used the prime intermittent dose method of urea kinetics (see section 3.1). During the final 24 hours of each study period, urea kinetics were measured using prime\intermittent doses of [$^{15}N^{15}N$] urea (99% atoms ^{15}N; Cambridge Isotope Laboratories, Cambridge, MA, U.S.A). An accurately measured amount of isotope was made up in sterile water and stored at 4°C until use. At 00.00 hours, a priming dose of isotopic urea equivalent to 15 hours of intermittent doses (28.5mg) was given orally to shorten the time taken to achieve a plateau of enrichment in urinary urea. From 06.00 hours until 18.00 hours, single doses of urea (5.5mg) were administered at three hourly intervals. Urine was collected immediately before the administration of the prime dose of urea isotope and at three hourly intervals from 06.00 until 21.00 hours to coincide with the taking of the intermittent doses of isotope and three hourly intakes of food. This protocol is respresented schematically in Table 5.6A.

Table 5.4A Experimental Protocol on Day 5

Time	00	06	09	12	15	18	21
Isotope	Prime	X	X	X	X	X	-
Urine Collection	X	X	X	X	X	X	X
Food	-	X	X	X	X	X	-
Supplement	-	X	X	X	X	X	-

5.2.4 Urine Collection

Total urine collection occurred throughout the five day protocol period. For days 1-4 urine was collected as passed into a new container every 12 hours. After isotope

administration, urine was collected at three hourly intervals into separate containers to coincide with intermittent doses. Urine was collected into plastic bottles, 1 litre for 12 hourly collections and 500ml for 3 hour collections. The bottles contained 2ml 6M HCl/100ml volume which acted as a preservative.

In order to store urine samples until analysis, the volume of each sample was noted before 60ml of each urine sample was pipetted into labelled plastic containers and frozen at -20°C. Remaining urine was disposed of.

In order to reuse the bottles, they were first rinsed many times with hot water, soaked over night in bleach before re-rinsing in a lot of hot water and finally rinsed in distilled water. The large volume of water used ensured that no residual isotope remained for the next use.

5.2.5 Analyses

The nitrogen content of the urine was measured by Kjeldahl analysis in 12 hour samples for days 1 and 4, and a pooled day 5 sample (section 3.3). The concentration of urea and ammonia in each 3 hour urine sample and the plasma sample were determined by the Berthelot Method (section 3.2). Urea nitrogen was isolated from urine for mass spectrometry using short ion-exchange column chromatography (section 3.4). Nitrogen gas was liberated from urea by reaction with alkaline hypobromite (section 3.5). In this reaction, N_2 is released from urea in a monomolecular reaction (Walser 1954); hence the relative proportions of [$^{15}N^{15}N$]urea, [$^{15}N^{14}N$]urea and [$^{14}N^{14}N$]urea can be determined (section 3.6). Measurements were carried out in a triple collector isotope ratio mass spectrometer (SIRA 10, VG Isogas, Winsford, Cheshire, U.K.). Inorganic sulphate (section 3.7) was also measured in the urinary samples.

5.3 Results

5.3.1 Anthropometry

All studies were completed satisfactorily and diets consumed as planned. Anthropometrical measurements from the subjects are shown in Tables 5.4 and 5.5. The energy intake of 126 KJ/kg/day was adequate to maintain body weight in all subjects.

Table 5.4 Measured Anthropometrical Data for the Six Adult Males.

	AGE Yrs	WT kg	HT cm	BI mm	TRI mm	SUB SCAP mm	SUP RAIL mm	MAC mm
1	21	72.2	191.5	5.4	10.5	8.5	3.0	255
2	21	71.1	179.6	5.0	5.1	8.5	3.9	285
3	23	83.4	178.8	6.8	9.3	15.8	6.8	310
4	22	84.6	181.3	11.4	9.3	10.3	3.7	305
5	22	71.4	183.3	14.3	12.5	12.3	5.4	285
6	22	80.4	178.2	10.5	11.8	12.5	5.2	330

WT = weight
HT = height

Skinfolds: BI = biceps
TRI = triceps
SUBSCAP = subscapular
SUPRAIL = suprailiac
MAC = mid arm circumference

From these results % body fat and hence lean body mass can be calculated using the equations of Durnin (section 3.9). Also the body mass index (WT/HT^2) is stated in Table 5.5.

Table 5.5 Calculated Anthropometrical Data for the Six Adult Males.

	Body Mass Index	% Body Fat	Total Body Fat (kg)	Lean Body Mass (kg)
1	19.6	12.56	9.06	63.19
2	21.9	10.13	7.26	64.44
3	26.0	16.54	13.79	69.60
4	25.5	15.38	13.01	71.59
5	21.3	18.38	13.12	58.28
6	25.4	17.09	13.74	66.66

The subjects basal metabolic rate was measured using indirect calorimetry.

Table 5.6 Basal Metabolic Rate Data For the Six Adult Males.

	$\dot{V}O_2$ ml/min	$\dot{V}CO_2$ ml/min	RQ	REE kJ/day	E REQ kJ/day
1	263	233	0.89	7660	10724
2	249	222	0.89	7260	10164
3	292	253	0.87	8470	11858
4	259	215	0.83	7440	10416
5	251	227	0.90	7340	10276
6	244	214	0.88	7070	9898

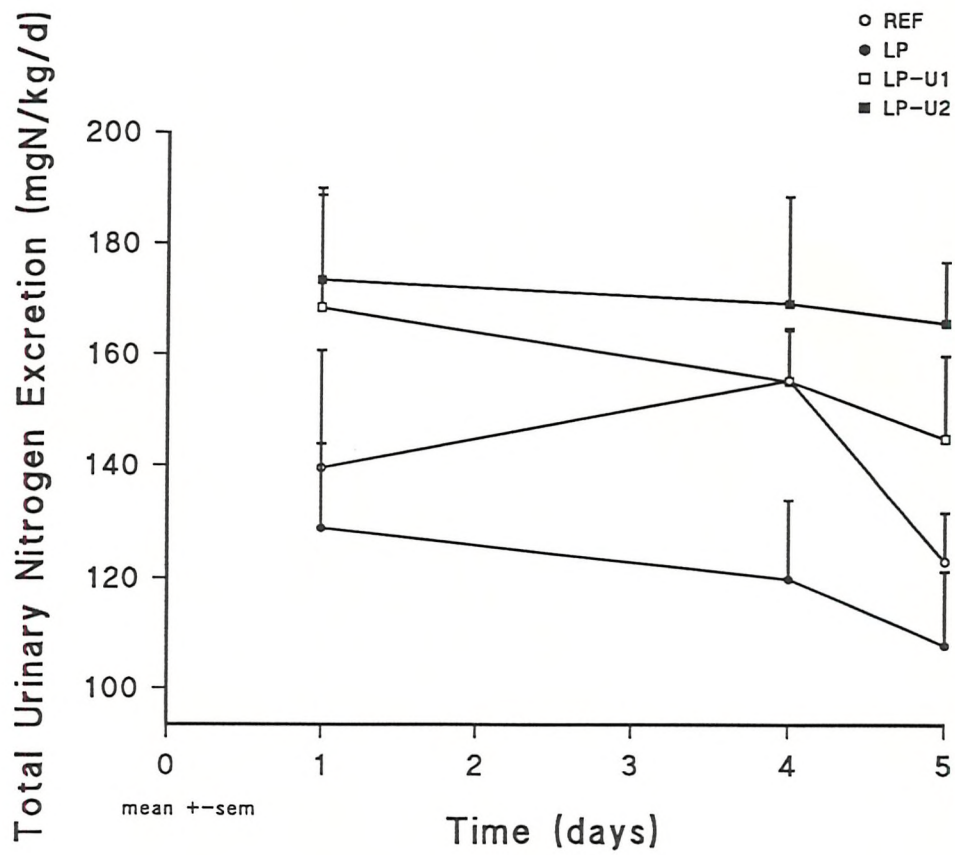
REE = resting energy expenditure

E REQ = energy requirement (REE x 1.4)

5.3.2 Urinary Nitrogen Excretion

Changes in nitrogen intake were reflected in urinary nitrogen excretion shown in Figure 5.1 (Individual results are shown in Appendix 3a). On the 70g protein/day diet, there was no significant difference in urinary excretion on any of the study days. On the 30g protein/day diet, nitrogen excretion fell from day 1 until reaching a reasonably steady metabolic state on day 5 when urea kinetics were measured. When urea was supplemented in the diet, nitrogen excretion was appreciably higher.

Figure 5.1 Urinary nitrogen excretion over the five day study period in six adult males fed diets with different nitrogen content.



Urea excretion followed the same patterns, being the major component of nitrogen loss in the urine. The relative proportions of nitrogen, urea and ammonia excretion over the five days of the study are shown in Table 5.7 (Appendix 3b).

Table 5.7 Urinary Excretion of Nitrogen, Urea and Ammonia in Six Adult Males in whom urea kinetics were measured on four occasions whilst consuming diets of different nitrogen content (mean ±sem).

INTAKE EXCRETION mgN/kg/d	REF 11.2gN	LP 4.8g N	LP-U1 8.0g N	LP-U2 11.2g N
NITROGEN				
DAY 1	140 ±21	129 ±15	168 ±20	173 ±17
DAY 4	155 ± 9	120 ±14	155 ± 9	169 ±19
DAY 5	123 ± 9	108 ±13	145 ±15	166 ±11
UREA				
DAY 1	111 ±19	122 ±15	205 ±35	155 ±19
DAY 2	118 ±17	100 ±14	137 ±20	143 ±17
DAY 3	126 ±13	79 ± 4	151 ±19	133 ±12
DAY 4	126 ±11	91 ± 9	131 ±12	111 ±13
DAY 5	125 ±12	77 ± 3	131 ±12	138 ±13
AMMONIA				
DAY 1	5.2 ±0.9	5.5 ±0.6	6.0 ±1.4	7.4 ±0.5
DAY 2	4.9 ±0.6	4.8 ±0.5	5.4 ±0.9	4.9 ±0.6
DAY 3	4.9 ±0.4	3.6 ±0.4	3.7 ±0.2	4.5 ±0.2
DAY 4	5.3 ±0.7	4.0 ±0.2	4.2 ±0.7	4.0 ±0.3
DAY 5	5.8 ±0.7	4.0 ±0.4	4.8 ±0.3	4.2 ±0.6

5.3.3 Apparent Nitrogen Balance

Apparent nitrogen balance was estimated by calculating the difference between nitrogen intake and loss. Nitrogen losses were taken to be total nitrogen lost in the urine over days 4 and 5, and an amount taken to be 24 mgN/kg/d (FAO 1985) for faecal and other endogenous losses (Table 5.8 and Appendix 3c).

There was no statistical difference between nitrogen balance achieved in the REF or the LP-U2 diets, nor between the LP or LP-U1 diets. Nitrogen balance was significantly less negative on the REF and LP-U2 diets than on the LP or LP-U1 diets. Thus, the addition of the lower level of urea supplement in LP-U1 (6.9g urea) did not improve nitrogen balance from the LP diet, but with the higher level of urea supplementation, LP-U2 (13.7g urea), nitrogen balance was significantly improved and not different to the REF diet.

Table 5.8 Apparent nitrogen balance in six adult males in whom urea kinetics were measured on four occasions whilst consuming diets of different nitrogen content (mean ±sem).

mgN/kg/d	REF	LP	LP-U1	LP-U2
INTAKE	146 ± 5	63 ± 2	104 ± 3	146 ± 5
URINE	140 ± 8	114 ± 7	150 ± 7	159 ±13
FAECAL & OTHER LOSSES	24	24	24	24
BALANCE	-18^{a} ±11	-76^{b} ± 5	-70^{b} ± 6	-37^{a} ±12

different superscripts indicate significant differences, $p<0.05$.

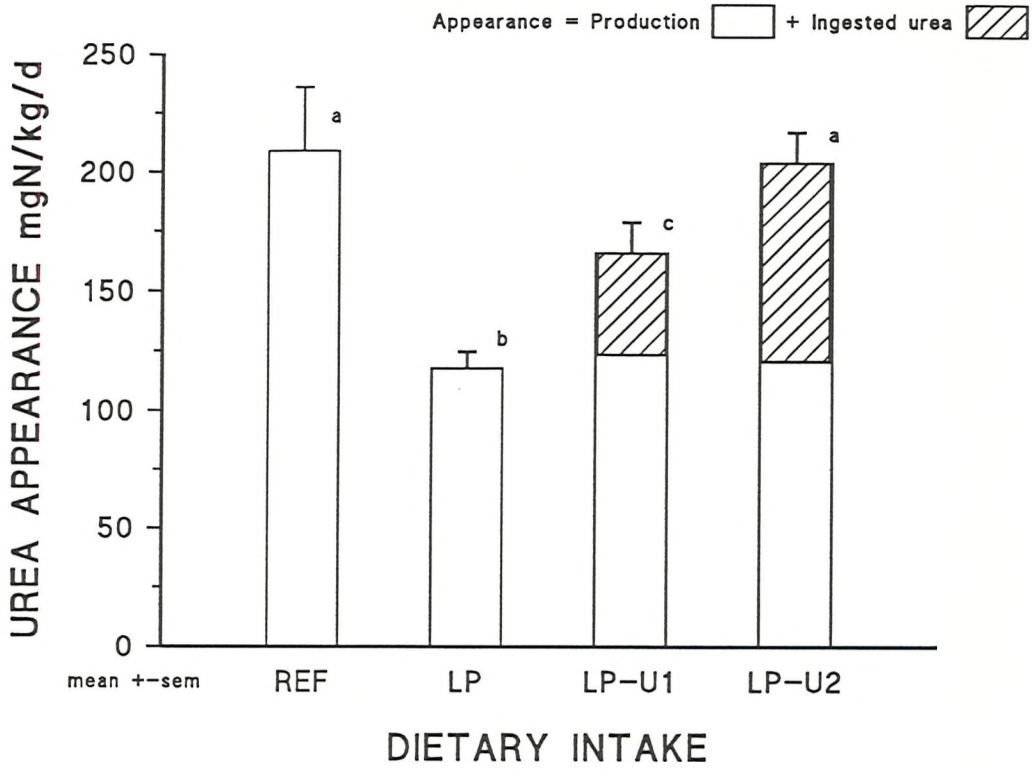
5.3.4 Urea Kinetics

Plateau enrichment of the double labelled urea isotope in urinary urea was identified by visual inspection and the average coefficient of variation at plateau was 14% for $[^{15}N^{15}N]$ urea and 19% for $[^{14}N^{15}N]$ urea.

Urea appearance (Au), measured by dilution of $[^{15}N^{15}N]$urea in the urea pool, was 209 $\pm$27mgN/kg/d on the REF diet. When intake was reduced to 30g protein/day (LP), appearance was significantly lower (118 $\pm$7mgN/kg/d), a value 40% lower than that of the higher protein intake. On addition of the two increasing urea supplements, appearance increased in a stepwise manner to 166 $\pm$13mgN/kg/d with 6.9g urea supplement and 204 $\pm$13mgN/kg/d with 13.7g urea supplement. These changes were significantly different to the LP diet. The diet with the 13.7g urea supplement provided the same quantity of nitrogen as the REF diet and appearance of urea in the body pool was not significantly different.

If the components Iu and Pu are considered, it can be seen that endogenous production is constant for all studies containing 30g protein in the diet, as shown in Figure 5.2. The increase in appearance is equivalent to Iu in diets supplemented with exogenous urea. For all low protein diets, the rate of endogenous urea production was about 60% of that on the REF diet, a significant difference in each case. Individual results for urea appearance and production are shown in Appendix 3d.

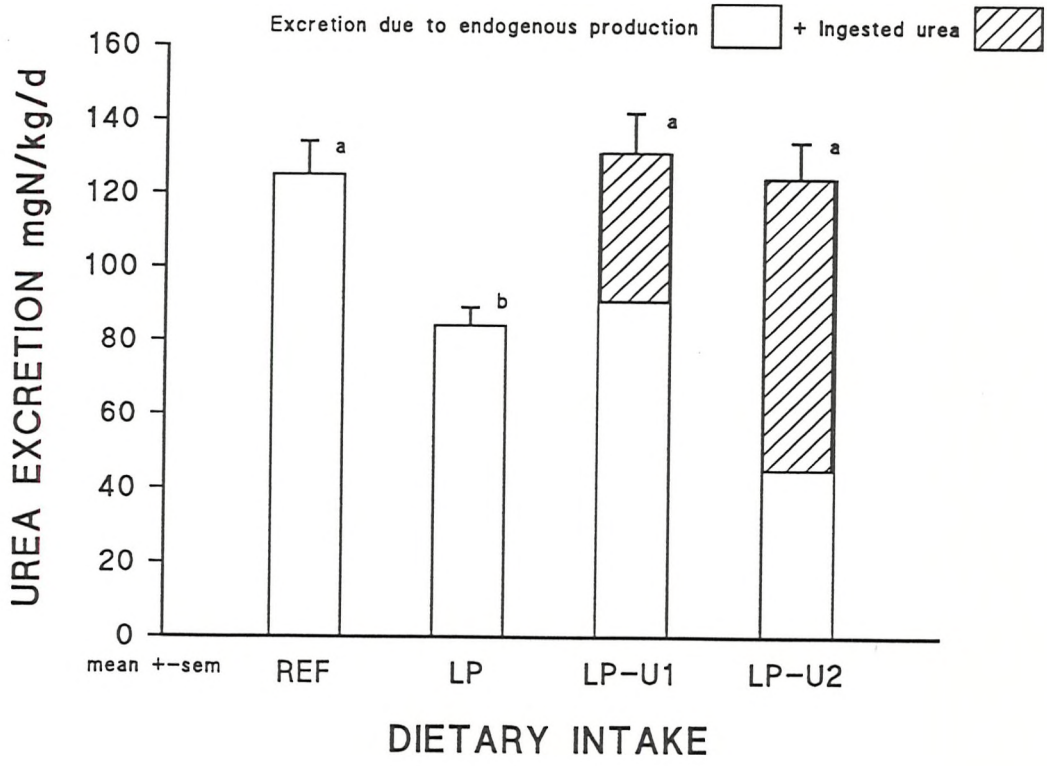
Figure 5.2 Appearance of urea in the body pool in six adult males consuming diets with different nitrogen content.



On the REF diet, urea lost via urinary excretion was 125 ±9mgN/kg/d. When nitrogen intake was reduced below minimum requirement levels on the LP diet, urea excretion fell to 84 ±5mgN/kg/d, a decrease of 33%. With urea supplementation, excretion rose to 131 ±11mgN/kg/d on the LP-U1 diet and 124 ±10mgN/kg/d on the LP-U2 diet. Individual results are shown in Appendix 3b. There was no significant difference in the rate at which urea was excreted in the urine between the REF diet and either of the diets in which urea was supplemented, LP-U1 and LP-U2, and there was no difference between urea excretion on the LP-U1 and LP-U2 intakes. Urea excretion was significantly greater on the REF, LP-U1 and LP-U2 diets than on the LP diet. Thus, although the addition of urea to the

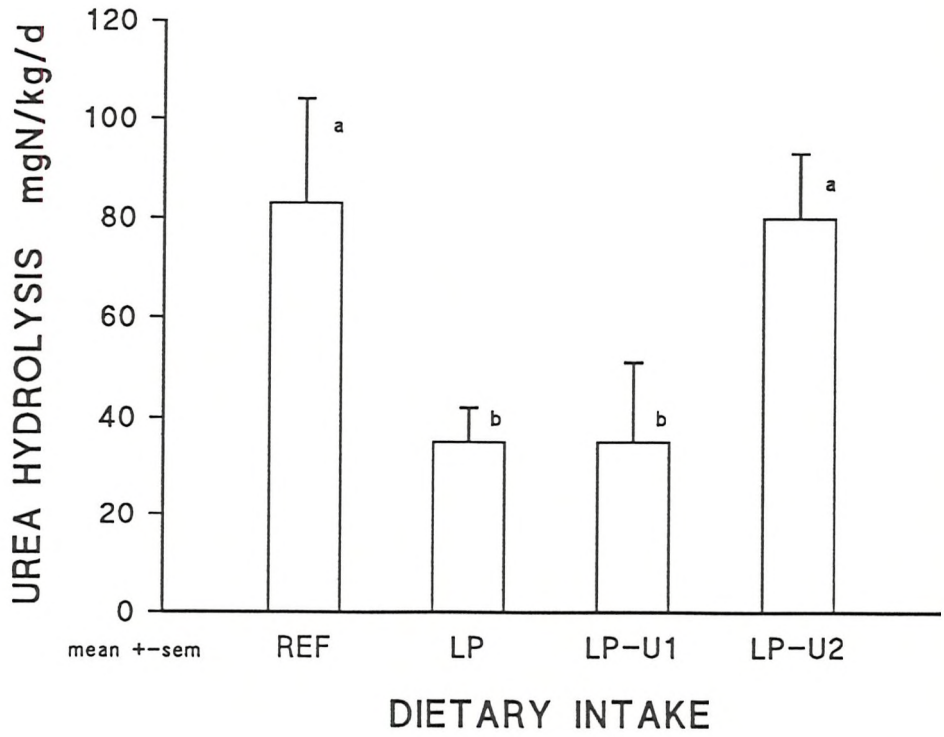
LP diet at the lower level resulted in an increase in urea excretion, equivalent to the dietary addition, the further addition at the higher level did not result in any further increase in excretion. This point is illustrated in Figure 5.3.

Figure 5.3 Urinary urea excretion in six adult males consuming diets with different nitrogen content.



From the assumption that the difference between urea production and that excreted over a 24 hour period (to allow for an expanding and contracting urea pool) has passed to the colon, hydrolysis (T) was calculated. As seen in Figure 5.4, hydrolysis on the REF intake was variable but had a mean value of 83 ±21mgN/kg/d, falling significantly to less than half (35 ±7mgN/kg/d) when only 30g protein/day was ingested, LP. There was no consequent increase in colonic hydrolysis with the LP-U1 diet (35 ±16mgN/kg/d) and this value was also significantly less than urea hydrolysis on the REF diet. With the LP-U2 diet, the amount of urea hydrolysed in the colon increased to 80 ±13mgN/kg/d. Individual results are shown in Appendix 3f.

Figure 5.4 Urea hydrolysis in the colon in six adult males consuming diets with different nitrogen content.



Urea hydrolysis on the LP-U2 diet was significantly increased compared to the LP and LP-U1 diet, and was not significantly different to the REF diet. Therefore, urea hydrolysis was reduced on the LP diet and the addition of the lower level of urea, LP-U1, did not change the rate of hydrolysis. However, with LP-U2, the addition of urea to the LP diet to bring the level of total nitrogen intake to that on the REF diet, returned the level of urea hydrolysis to a rate not different to the REF diet.

The values for the amount of nitrogen from hydrolysis returning to the body pool via synthetic pathways (S) and that passing back into urea production (Pr) are shown in Table 5.9 (Appendix 3g). On each of the diets, about 14% of the hydrolysed urea-nitrogen returned to the urea pool. The nitrogen retained in the body via synthetic pathways was over 80% of the nitrogen arriving in the body pool from urea hydrolysis. There was no difference in nitrogen retention between either the REF and the LP-U2 diets, nor between the LP and LP-U1 diets, and retention on LP-U2 was significantly greater than on LP-U1.

Table 5.9 Urea kinetics for six adult males consuming diets with different nitrogen content (mean ±sem).

mgN/kg/d	REF	LP	LP-U1	LP-U2
Intake	146^{a} ± 5	63^{b} ± 2	104^{c} ± 3	146^{a} ± 5
Appearance	209^{a} ±27	118^{b} ± 7	166^{c} ±13	204^{a} ±13
Production	209^{a} ±27	118^{b} ± 7	124^{b} ±14	121^{b} ±13
Excretion	125^{a} ± 9	84^{b} ± 5	131^{a} ±11	124^{a} ±10
Hydrolysis	83^{a} ±21	35^{b} ± 7	35^{b} ±16	80^{a} ±13
Urea Formation (Pr)	12.0^{a} ±2.8	6.0^{b} ±1.0	5.2^{b} ±0.9	8.0^{a} ±0.9
Synthetic Pathways (S)	71^{a} ±20	29^{b} ± 7	30^{b} ±16	72^{a} ±12

different superscripts indicate significant differences, $p<0.05$.

5.3.5 Clearance

The concentration of urea in plasma was measured at 15.00 on day five of the study. Urea pool size was calculated from plasma urea values multiplied by total body water (Watson 1980) and correlated to clearance of urea via excretion (Eu) and via the bowel (T). These results are shown in Table 5.10 and Appendix 3h.

Table 5.10 Urea pool size and clearance of urea from the pool by excretion or hydrolysis in six adult males consuming four diets with different nitrogen content (mean ±sem).

Intake	REF 11.2g N	LP 4.8g N	LP-U1 8.0g N	LP-U2 11.2g N
Plasma Urea (mg/100ml)	17.6^{a} ± 0.9	16.5^{a} ± 1.6	21.0^{b} ± 0.8	27.0^{c} ± 2.2
Urea Pool (g)	8.1^{a} ± 0.5	7.1^{a} ± 0.5	9.6^{b} ± 0.4	12.3^{c} ± 1.0
Renal Clearance (ml/min)	38.8^{a} ± 3.8	27.9^{b} ± 3.1	33.0^{ab} ± 1.4	26.9^{b} ± 4.5
Bowel Clearance (ml/min)	25.7 ± 7.2	12.0 ± 1.9	9.2 ± 4.3	14.0 ± 1.8

different superscripts indicate significant differences, $p<0.05$.

Equations for these calculations are:-

Urea Pool Size (g) = Plasma Urea Conc (gN/1000ml) x Total Body Water (litres)

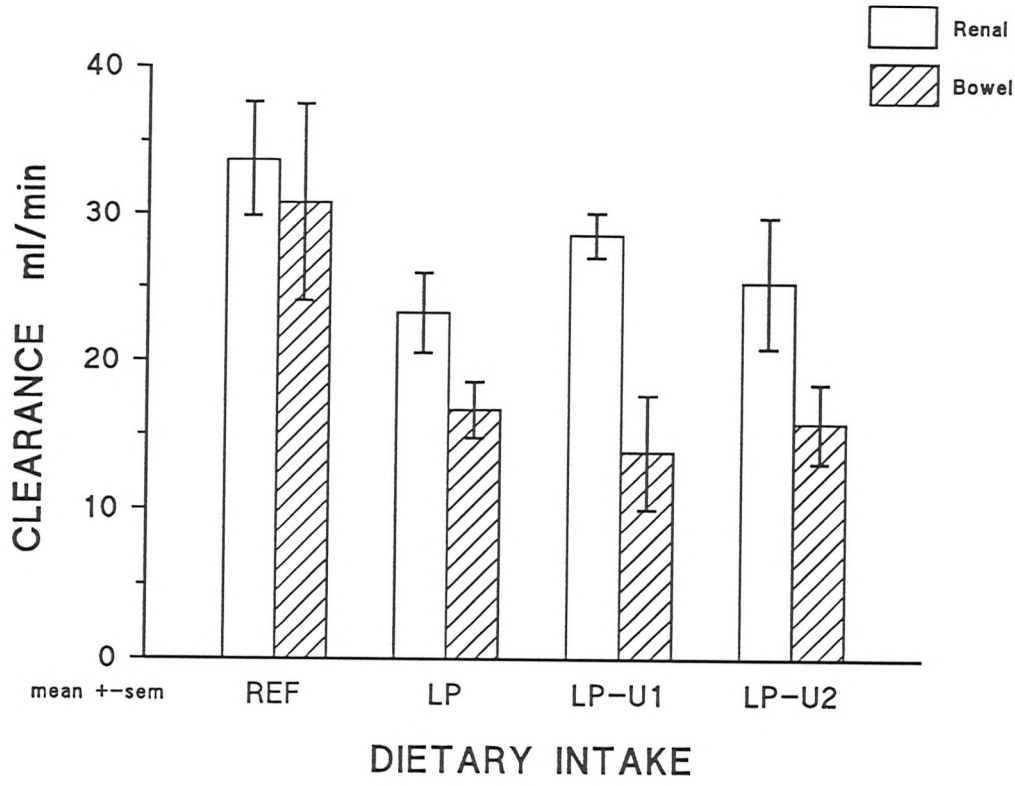
Total Body Water (l) = 2.447 - 0.9516 Age (yrs) +0.1074 Ht (cm) +0.3362 Wt (kg)

Renal Clearance (ml/min) = $\frac{\text{Rate of Excretion}}{\text{Plasma Conc}}$ (urine conc x flow)

Bowel Clearance (ml/min) = $\frac{\text{Rate of Hydrolysis}}{\text{Plasma Conc}}$ (T)

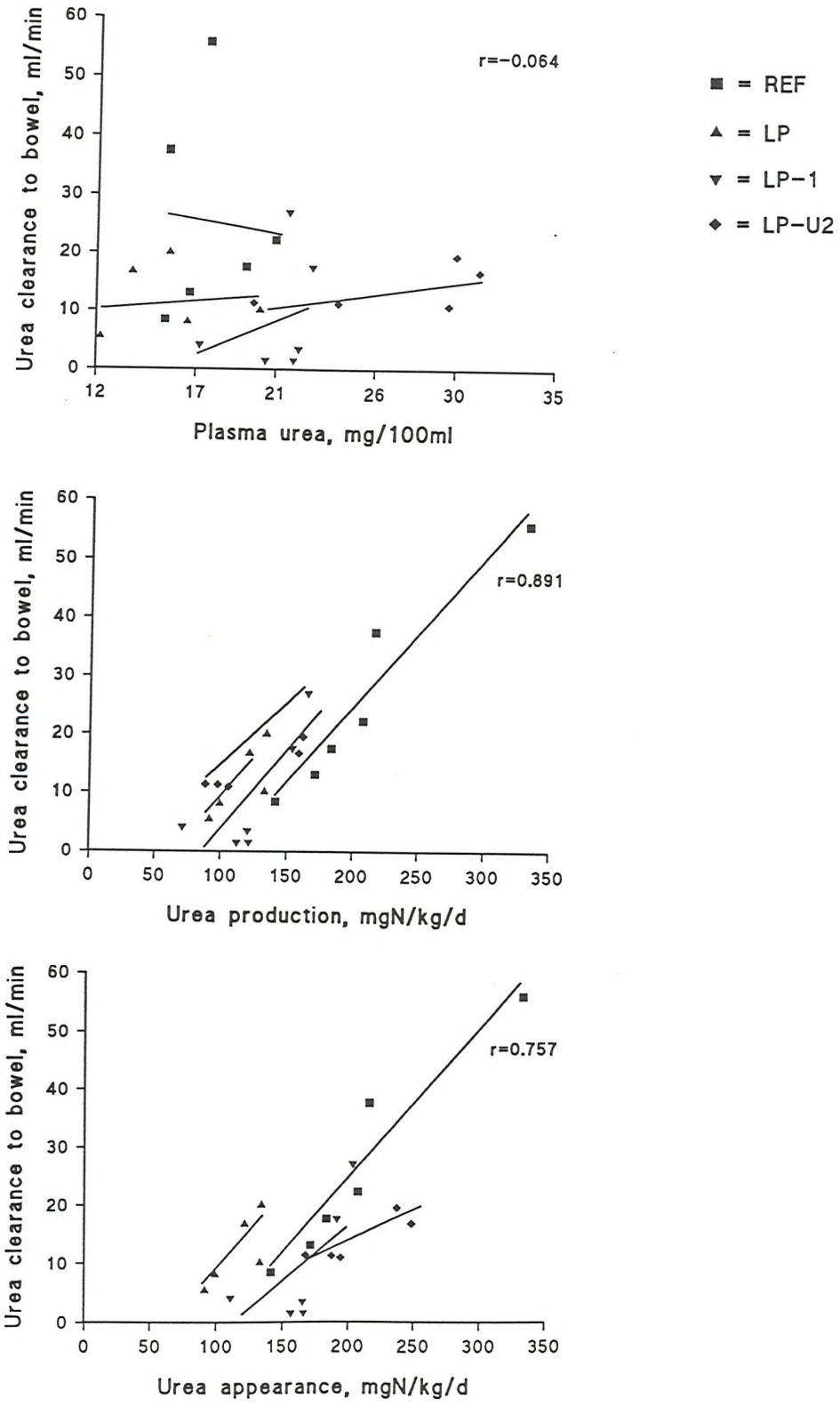
There was no significant difference in the plasma urea concentration and urea pool between the reference 70g protein/day diet, REF, and the LP diet with a 30g protein/day intake. There was a significant increase in pool size on the LP-U1 diet, and a further significant increase on the LP-U2 diet. Renal clearance of urea was significantly lower on the LP and LP-U2 diets than the REF diet. The LP-U1 diet was significantly different to the LP-U2 diet but did not reach statistical difference to the LP diet. The clearance of urea into the bowel was about half of that on the REF diet on each of the low protein diets, but because of variability, the only difference of statistical significance was between the REF and the LP-U1 diet. Clearance into the bowel was about 66% of clearance into the urine for the REF diet, whereas it was only about 28-52% for the LP, LP-U1 and LP-U2 diets. The relative proportions of urea clearance in the urine or to the bowel are represented in Figure 5.5.

Figure 5.5 Relative proportions of renal and bowel clearance in six adult males consuming diets with different nitrogen content.



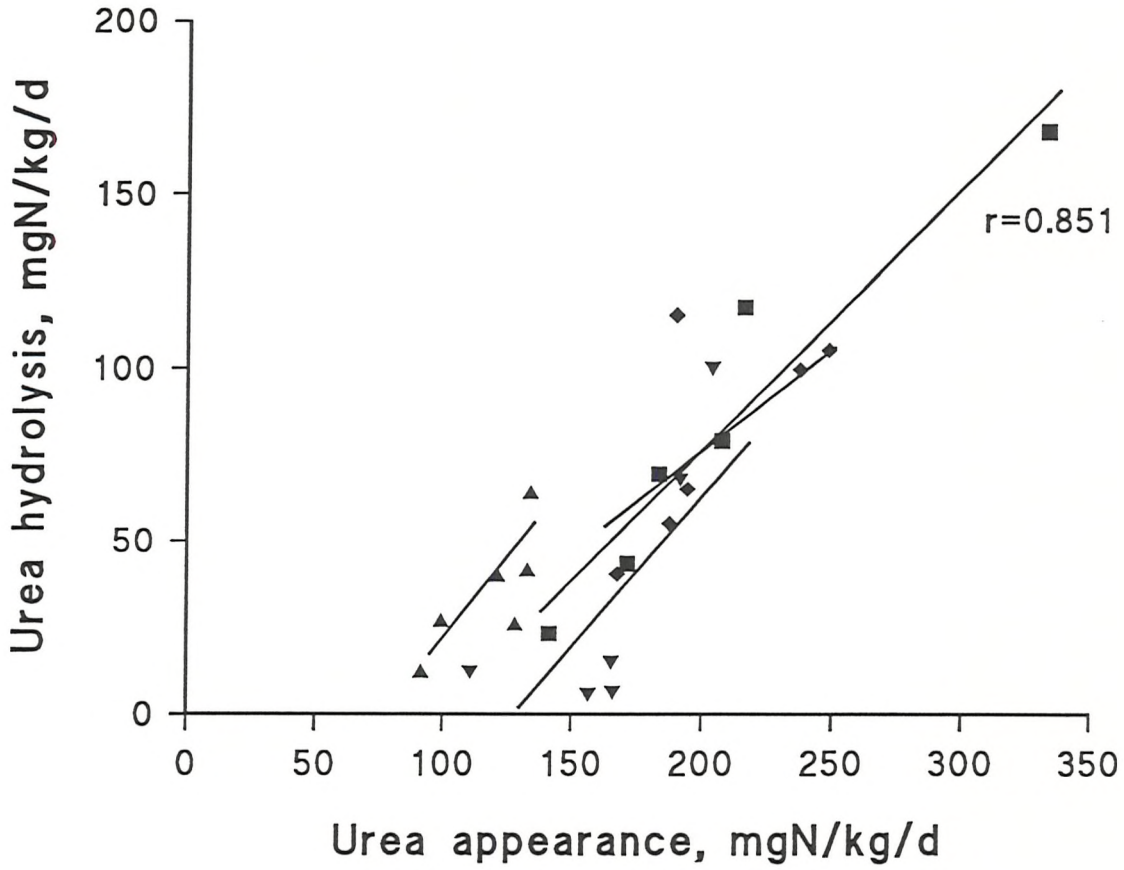
The relationships between clearance into the urine or bowel with plasma urea concentration, pool size and urea production rates were investigated. For renal clearance, no statistical relationships were found, except for very weak associations with urea production (r=0.571) and with plasma urea (r=-0.40). For clearance in the bowel, there was no relationship with either plasma urea concentration or the urea pool size. However, Figure 5.6 demonstrates the relationship between urea clearance into the bowel and urea appearance, showing a very strong statistical association.

Figure 5.6 Relationships between urea clearance to the bowel and other metabolic factors measured in six adult males on four different nitrogen intakes (linear regression analysis).



The variables used to calculate urea clearance into the bowel are the plasma concentration of urea and urea hydrolysis. From Figure 5.7, it can be seen that urea hydrolysis is directly related to urea appearance (r=0.86).

Figure 5.7 The relationship between urea hydrolysis and the rate of urea appearance in six adult males consuming diets with different nitrogen content.



5.4 Discussion

The purpose of this study was to determine the response when a non-essential nitrogen source, in the form of urea, was supplemented to a diet below minimal requirements for protein. The method of urea kinetics was utilised to ascertain whether the microflora inhabiting the colon would hydrolyse this exogenous supply and make the nitrogen available for metabolic processes. The results show that salvaging in the large intestine does increase when a source of dietary non-essential nitrogen is introduced and that the overall nitrogen status of the individual improves as the hydrolysed urea is incorporated into the metabolic nitrogen pool of the body. However, these changes only occurred when substantial quantities of exogenous urea were added to the diet, an amount equivalent to nitrogen available from 40g protein. This leaves the question of what other factors may be influencing the control of this salvaging system.

In the past, studies examining the role of non-essential nitrogen have used the measurement of nitrogen balance which, although useful, does have its limitations. There is the possibility of overestimation of nitrogen retention indicated by unrealistic positive balances and the length of the study period is important as nitrogen balance can eventually be achieved over a range of nitrogen intakes. Urea kinetics provides a quantitative approach which begins to clarify the mechanism behind the utilisation of non-essential nitrogen. The prime-intermittent oral dose method is a simple non-invasive procedure which allows the direct measurement of the rate of appearance of endogenous urea with out the susceptibility to error caused by incomplete urine collection in the single dose method (Jackson *et al* 1993b). The four diets in the study were designed to challenge the hypothesis directly. The 70g protein/day intake was a reflection of an adequate daily intake for adult males in the UK (Gregory *et al* 1990). The 30g

protein/day intake was purposely below requirement levels at a level where, as shown in Danielsen 1992, salvaging fails to maintain nitrogen balance as adaptive mechanisms described by Waterlow (1968) are unable to meet the body's demand for nitrogen.

The quantity of exogenous urea supplied was calculated from the amount of nitrogen required to replace half and then all of the shortfall in nitrogen intake with reduction to 30g protein/day from 70g protein. It was ensured that energy requirements were met as it has been shown that when insufficient calories are present for the synthesis of non-essential amino acids, then supplementary non-essential nitrogen has no beneficial effect on nitrogen balance (Gallina & Dominquez 1971) and if calorie intake is increased, nitrogen retention is improved (Anderson *et al* 1969). It was decided to use habitual food rather than a liquid diet intake for several reasons. Despite a slight lowering of confidence in the accuracy of protein intakes, a solid food diet was preferential to a liquid formula which may have influenced colonic function, although addition of non-starch polysaccharide to a liquid feed has been shown not to influence urea kinetics (Langran *et al* 1992). Subject compliance, however, is more likely to be enhanced when the food prepared was enticing and as near to habitual intake as possible.

In order to be able to measure urea kinetics with reliability presumes a reasonably steady metabolic state is achieved. After considering other studies (Rand *et al* 1976, Langran *et al* 1992, Danielsen & Jackson 1992, Quevedo *et al* 1994), it becomes clear that although it might take some time to achieve a full adaptive response on moving from one level of protein intake to another, much of the change has taken place within three to four days, by which time, a reasonably steady state has been established.

Nitrogen balance was estimated using nitrogen losses in the urine and an amount stated in the 1985 FAO report to be

that lost from faeces and other endogenous losses such as skin, hair and secretions, based on the data from Calloway 1971. Faecal nitrogen excretion does not alter with supplemental urea intake (Korsland *et al* 1977). The data presented confirm that on a diet which provides 30g protein/day, normal men are unable to maintain nitrogen balance. A significant improvement of apparent nitrogen balance was seen with the addition of the larger supplement of urea. The results were consistent with numerous experiments which have demonstrated an improvement in nitrogen balance with non-essential nitrogen supplements (Clarke *et al* 1963, Kies *et al* 1972b, 1978). However, it was surprising that there was no improvement with 6.9g urea as this is still a substantial amount of nitrogen (3.2g is equivalent to 20g of protein). It appears that balance can only be achieved when total nitrogen intake exceeds a threshold somewhere between 8-11gN/day. On examination of the urea kinetics, some insight can be gained into this observation.

Urea metabolism has two control points. The site of hepatic production and the proportion of the urea produced which is excreted compared to the proportion which passes to the colon where the nitrogen is salvaged (Picou & Phillips 1972). The most important elements of the study can be grasped by considering the components of the system. Appearance was measured directly by dilution of labelled [$^{15}N^{15}N$] urea and is a result in which there is the greatest confidence. The stepwise rise from the value achieved on the LP diet to those obtained on the supplemented diets reflected directly the quantitative intake of exogenous urea. Appearance of urea in the body pool directly correlated with nitrogen intake, from whatever source; the appearance rate from the REF diet resembling that measured when 30g protein with 13.7g urea was ingested. It is a conclusive validation of the accuracy of measuring production. Proportionate amounts of urea are absorbed into

the bloodstream and are reflected in the appearance of urea in the urea pool. When appearance due to exogenous urea (Iu) was removed from the results, this give us the value for endogenous urea production (Pu), which is similar for all diets containing 30g protein, showing that all of the increase in appearance was due to ingestion of urea rather than an actual increase in hepatic production from amino acid catabolism. From this, it can be said that whilst appearance of urea in the body pool is related to the total nitrogen intake, endogenous urea production is governed by the intake of protein.

The fate of urea produced by hepatic enzymes, recycled from the nitrogen pool or ingested in the diet is either excretion or hydrolysis by bacteria in the colon. The proportion that goes to each is determined by indefinite factors. On reduction to the low protein diet urea excretion fell in order to conserve body nitrogen, but intake was below minimal and hence, exceeded the limitations of adaptation. With addition of non-essential nitrogen into the diet, excretion rose compared to the 30g protein value. However, when excretion due to this ingested urea (Iu) was accounted for, a different pattern from that of appearance in the body pool became obvious. Therefore, excretion is not simply a reflection of appearance or endogenous production and other determinants must be influencing this process. The results show a real decrease in the proportion of urea excretion estimated to be due to endogenous production on the LP-U2 intake. However, overall nitrogen excretion did not fall to such an extent so perhaps other nitrogenous compounds were excreted in preference. So, when exogenous urea is removed from the data, production stays equal to that when 30g protein is ingested but less is excreted. The answer is uncovered when hydrolysis results are considered. There is a subsequent rise in hydrolysis of urea on the LP-U2 diet. The body is utilising this non-essential nitrogen source to improve nitrogen balance and the diet is no longer

sub-requirement.

Nonsporing anaerobes are the major source of ureases in man. It has previously been observed that the proportion of those microorganisms that produce ureases increases with blood urea concentration (Brown *et al* 1971). Hence, it was thought the quantity of nitrogen recycled from urea hydrolysed in the colon depends on the concentration of urea in body fluids. However, in this study no correlation was found between rate of hydrolysis and urea pool size. This may have been because the system was operating at the minimum levels for adaptation. However, when 6.9g supplemental urea was added there was no increase in hydrolysis for a large expansion of the body's urea pool and so the conclusion could be drawn that the urease containing colonic bacteria do not hydrolyse urea on a concentration dependent basis.

The ability to enhance urea hydrolysis as urea concentration falls on lowering protein intake, therefore requires further consideration. This study demonstrates a close relationship between urea clearance into the bowel and the rates of urea production and appearance. The calculation of urea clearance to the bowel is based upon two components; the plasma urea concentration and the rate of urea hydrolysis. As there was no association between clearance to the bowel and plasma urea levels, the relationship between urea hydrolysis and rate of urea appearance in the pool were examined and found to be very close. Therefore, the higher the rate of appearance, the greater the clearance, or the less urea cleared in a given time for a fixed blood flow. Under these circumstances, it can be concluded that urea hydrolysis is driven by urea appearance.

So what is happening with the 6.9g urea supplement? This diet is insufficient to alter the deficit in nitrogen balance and hydrolysis of urea does not increase significantly above levels found on the low protein diet.

The body appears unable to respond to this input of nitrogen and utilize it via colonic salvaging. In order for urea nitrogen to result in non-essential amino acid synthesis, both the hydrolysis of urea and the incorporation of ammonia with carbon skeletons into non-essential amino acids must take place. Which of these two steps is restricted is unknown. Energy intake was sufficient at the BMR x 1.4 level but this may have not been sufficient for those leading a more active lifestyle, reflected in the marginally negative nitrogen balance on the REF and LP-U2 diets, and hence energy could potentially be a limiting factor. For some reason, it is not possible to incorporate this source of nitrogen into the body proteins. At this level of intake, the body is functioning on the boundaries of adaptational limitations. Other dietary factors may be limiting in this circumstance or the function of the microflora may be compromised. It has also been shown that urea is not the most efficient source of non-essential nitrogen and other forms, such as glycine or ammonium salts may have an increased effect (Rose 1949, Swendseid *et al* 1960, Kies *et al* 1972b, 1974). The influence of glycine on urea-nitrogen salvage is examined in Chapter 7.

On consideration, it seems possible that the limiting factor in this system may be sulphur. When the amino acid score of the 30g protein/day (LP) diet is examined (shown in Appendix 1d), it can be seen that methionine is the limiting amino acid. Hence, in retrospect, the sulphur excretion in the urine was measured as inorganic sulphate and results can be seen in Table 5.11. The results indicate that the utilisation of urea nitrogen by the colonic bacteria may be influenced by sulphur in some way. Excretion of sulphate can be increased just by addition of non-essential nitrogen. The exploration of this will form the basis of the next chapter in this thesis.

Table 5.11 Urinary inorganic sulphate excretion in six adult males consuming diets with different nitrogen content (mean ±sem).

	REF 11.2g N	LP 4.8g N	LP-U1 8.0g N	LP-U2 11.2g N
Inorganic Sulphate Excretion mmol/d	22.9 ±1.9	19.6 ±0.6	23.4* ±1.1	22.0 ±2.2

* significantly different to the LP diet.

The implications of this study bring the ideas of protein requirements into question. It can no longer be ignored that non-essential nitrogen is a limiting factor and this nitrogen source has rightly been incorporated into the model for protein requirements (Millward & Rivers 1988). Non-essential nitrogen may be especially beneficial when dietary protein intake is restricted, as with malnutrition, the importance of nitrogen source in breast milk for infant nutrition or the treatment of chronic renal failure (Brenner 1982). Another important factor is the contribution of the colon to protein nutrition, increasingly so when total intake is restricted.

Nitrogen balance and isotopes studies have shown that nitrogen from inorganic sources, such as urea, may be incorporated into body protein, entering the pool of nitrogen available for synthesis of amino acids or urea via microbial hydrolysis in the colon. This present study supports conclusions that the utilisation of non-essential nitrogen is enhanced when dietary intake of nitrogen is insufficient. The technique of urea kinetics allows an insight into the mechanism behind the improvement of nitrogen balance when an exogenous source of urea is added

to a diet below minimal requirements for protein. However, the observed increase in hydrolysis by the colonic microflora is restricted until substantial amounts of urea are present and hence, the next question is to determine the factors controlling this process.

5.5 Summary

This study shows that a diet which provides 30g protein/day is not adequate, but addition of large amounts of nitrogen in the form of urea can produce improved nitrogen balance, through the enhanced salvaging of urea nitrogen. As urea production appeared related to protein intake, but urea salvage was related to the rate of appearance of urea nitrogen, we would conclude that the availability of urea is an important determinant of urea salvage. The urea available for excretion in urine is that which is not salvaged, thus the active process appears to be urea salvage and hence, the ability to maintain urea salvage appears to be a critical mechanism through which nitrogen balance is maintained.

Therefore, nitrogen from urea can be incorporated into the body nitrogen pool and may be particularly important when the dietary intake of nitrogen is restricted. At the boundary of successful adaptation, relatively large amounts of urea were required to improve apparent nitrogen balance, suggesting there may be other limiting factors.

CHAPTER 6

THE INFLUENCE OF SULPHUR AMINO ACIDS ON UREA-NITROGEN SALVAGE.

In the preceding chapter, it was found that exogenous urea was utilised by colonic bacteria when dietary protein intake was below minimal requirement. However, large quantities of this non-essential nitrogen source were required to produce this effect, leaving a question as to whether other limiting factors were important. The purpose of this study was to determine whether the bioavailability of sulphur amino acids is a contributing factor to this system.

6.1 Introduction

The body does not use sulphur itself as a nutrient but it occurs in important nutrients such as thiamin and sulphur amino acids. Sulphur is necessary for protein structure and hence enzyme function, extracellular matrix composition and removal of potentially dangerous compounds from the body via detoxification reactions or quenching of free radicals by glutathione.

On examination of the experiments looking at the effects of non-essential nitrogen on essential amino acid requirements, the importance of the influence of sulphur amino acids and in particular methionine becomes apparent. Rose's pioneering investigations into dietary amino acids led to the classification of the sulphur containing amino acid methionine as being indispensable (Rose 1938, 1955). From these experiments, a series of studies followed in order to determine the daily requirement for this nutrient (Reynolds *et al* 1958, Swendseid *et al* 1956, Clarke *et al* 1970). Interactions between methionine and its derivative cysteine, the total amount of nitrogen present in the diet provided and inaccuracies of the

nitrogen balance technique led to conflicting estimates. The current accepted value is 900mg methionine per day (FAO 1985). Following this criterion, the 30g protein diet used in the previous study was limiting in methionine, and hence, sulphur. In fact, the sulphur containing amino acids have been found to be the first limiting amino acids in several important food sources such as potatoes, soy beans and legumes (Kies & Fox 1975). It has also been demonstrated that in milk protein (Snyderman *et al* 1962) and corn protein (Kies & Fox 1972b), the next limiting factor after non-essential nitrogen is the availability of methionine. Of wider implication, methionine may be the limiting factor of greatest importance in the nutrition of developing countries.

Nitrogen and sulphur metabolism are inevitably linked, both being constituents of protein. The level of total dietary nitrogen influences the order in which amino acids become limiting in the dietary protein source (Kies & Fox 1970a) and when protein intake is lowered, the sulphur amino acids become limiting. Nitrogen balance on these intakes can be improved using methionine supplements (Kies & Fox 1975, 1978, Korsland *et al* 1973, Zezulka & Calloway 1976). However, some investigators have not seen this beneficial effect, possibly due to the level of nitrogen intake being relatively high (Clarke & Woodward 1966, Kaneko *et al* 1986). Young saw no improvement in nitrogen balance with additional methionine when nitrogen intake was 128mgN/kg/d but did at a nitrogen intake level of 82mgN/kg/d (Young *et al* 1984). It has also been noted that fortifying soy based infant formulas with methionine improved nutritional status in early life (Fomon *et al* 1979).

Various suggestions have been put forward to explain this beneficial effect of sulphur amino acids. Methionine supplementation has been shown to increase whole body protein synthesis in chicks (Muramatsu *et al* 1986). The improvement in nitrogen balance may be due to decreased migration of free amino acids from peripheral tissues to the liver and the decreased level of liver ornithine, reducing endogenous urea formation in rats on a protein free diet supplemented with

methionine and threonine (Yokogoshi & Yoshida 1977). However, it is the aim of this study to examine whether this phenomenon can be explained by the activity of the colonic microflora.

The previous study demonstrated that the nitrogen source could influence changes in urinary sulphur excretion, indicating an effect on sulphur amino acid metabolism. The colonic bacteria responsible for recycling of nitrogen from the large intestine can themselves be influenced by methionine analogues which appear to increase the urease activity (Smith *et al* 1979) and this could be a possible mechanism for increasing the salvage of urea nitrogen.

Hence, the purpose of this study was, firstly, to determine whether methionine is a limiting factor in our low protein diet by supplementing this amino acid at levels such that requirement values are met and to determine the nitrogen status of the subjects using urea kinetics. Secondly, this technique will be employed to determine whether the limitations in utilisation of non-essential nitrogen sources by the colonic microflora are overcome by addition of a supply of bioavailable sulphur, in the form of methionine.

6.2 Protocol and Methods

6.2.1 Protocol

The studies were carried out in six normal adult females aged 22-28. The subjects agreed to participate after the nature of the study had been explained to them. They were all in good health at the time of the studies. The study had the approval of the Southampton Hospitals and South West Hampshire Health Authority Ethical Subcommittee. Each subject underwent four protocols (Table 6.1), each of which lasted five days. For each of the protocols, the subject was provided with a standard diet to eat over the five day period. Each subject had her basal metabolic rate measured by indirect calorimetry along with other anthropometrical data. Complete collections of urine were made for the duration of the study, and during the last 24 hours of each study period measurements of urea kinetics were made. A plasma sample was taken at 3pm on day 5 and stored at -70°C. The studies were carried out in the order shown and at the same stage in the menstrual cycle (week 4, the luteal phase) and hence,three weeks passed between consecutive studies.

Table 6.1 The Dietary Protocol

DIET	PROTEIN	METHIONINE	UREA	TOTAL NITROGEN
LP	26g	---	---	4.16g
LP-M	26g	600mg	---	4.22g
LP-U	26g	---	6.9g	7.36g
LP-UM	26g	600mg	6.9g	7.42g

6.2.2 Diets

The low protein diet (LP) was fed at a level of 26g protein/day, as this was 0.42g/kg/d, an equivalent amount to the 30g protein/day diet in the male subjects in the previous study. The diet was again whole food intake rather than liquid formula and calculated using food tables (McCance and Widdowson 1991). The structure of the diet is shown in Table 6.2 and appendix 1C. The 26g protein diet was found to contain 358mg methionine, using the Comp-eat computer analysis (appendix 1D). If it is assumed that the methionine is 85% available, then 304mg is delivered to the body. Hence, to make the level of methionine up to the requirement level of 900mg, it is necessary to supplement an extra 600mg/day (LP-M). The smaller urea dose, 6.9g, as supplemented in the previous study was supplemented, alone or in addition to methionine, in the third (LP-U) and fourth (LP-UM) dietary protocols respectively, in order to see if addition of sulphur would improve the utilisation of this nitrogen source. The supplements were given in the form of a flavoured drink, taken with meals. Food was eaten ad libitum on days 1-4, but on day 5, food was eaten at three hourly intervals to coincide with isotope administration.

Table 6.2 The 26g Protein Diet

	PORTION SIZE (g)	PROTEIN CONTENT (g)	ENERGY CONTENT (kJ)
RICE KRISPIES	30	1.6	466
WHOLE MILK	120	3.1	336
ORANGE JUICE	100	0.5	139
JAM SANDWICH	110	5.5	1478
HULA HOOPS	35	1.2	218
APPLE	120	0.2	176
JAFFA CAKES	20	0.7	307
BOILED SWEETS	65	---	895
LUCOZADE	250	---	714
MEALS :- CHOICE OF			
1. VEGETABLE CURRY WITH RICE	300	7.2	1231
SALAD AND DRESSING	200	1.8	349
PEACHES AND CREAM	180	1.4	1079
2. VEGETABLE LASAGNE	300	8.4	1012
SALAD AND DRESSING	200	1.8	349
PINEAPPLE AND CREAM	180	1.4	1134
TOTAL		23.8	8433

6.2.3 Urea Kinetics

The protocol used the prime intermittent dose method of urea kinetics (see section 3.1). During the final 24 hours of each study period, urea kinetics was measured using prime/ intermittent doses of [$^{15}N^{15}N$] urea (99% atoms ^{15}N; Cambridge Isotope Laboratories, Cambridge, MA, U.S.A.). An accurately measured amount of isotope was made up in sterile water and stored at 4°C until use. At 00.00 hours, a priming dose of isotopic urea equivalent to 15 hours of intermittent doses (28.5mg) was given orally to shorten the time taken to achieve a plateau of enrichment in urinary urea. From 06.00 hours until 18.00 hours, intermittent doses of urea (5.5mg) were administered at three hourly intervals. Urine was collected immediately before the administration of the prime dose of urea isotope and at three hourly intervals from 06.00 until 21.00 hours to coincide with the taking of the intermittent doses of isotope and three hourly intakes of food.

6.2.4 Urine Collection

Total urine collection occurred throughout the five day protocol period. For days 1-4 urine was collected as passed into a new container every 12 hours. After isotope administration, urine was collected at three hourly intervals into separate containers to coincide with intermittent doses. Urine was collected into plastic bottles, 2 litres for 12 hourly collections and 500ml for 3 hourly collections. The bottles contained 2ml 6M HCl/100ml volume which acted as a preservative.

In order to store urine samples until analysis, the volume of each sample was noted before 60ml of each urine sample was pipetted into labelled plastic containers and frozen at -20°C. Remaining urine was disposed of.

6.2.5 Analyses

The nitrogen content of the urine was measured by Kjeldahl analysis (section 3.3). The concentration of urea and ammonia in urine and plasma was determined by the Berthelot Method (section 3.2). Urea nitrogen was isolated from urine for mass spectrometry using short ion-exchange column chromatography (section 3.3). Nitrogen gas was liberated from urea by reaction with alkaline hypobromite (section 3.4). The relative proportions of $^{15}N^{15}N$ urea, $^{15}N^{14}N$ urea and $^{14}N^{14}N$ urea in the nitrogen gas evolved can be determined using a triple collector isotope ratio mass spectrometer (SIRA 10, VG Isogas, Winsford, Cheshire, U.K.). Inorganic sulphate (section 3.7) was also measured in the urinary samples.

6.3 Results

6.3.1 Anthropometry

All studies were completed satisfactorily and diets consumed as planned. Anthropometrical data is shown in Tables 6.3 and 6.4. The energy intake of 138 kJ/kg/d was adequate to maintain body weight in all subjects.

Table 6.3 Measured Anthropometrical Data for the Six Adult Females.

	AGE Yrs	WT kg	HT cm	BI mm	TRI mm	SUB SCAP mm	SUP RAIL mm	MAC mm
1	22	64.4	166.7	18.8	23.2	25.4	14.4	290
2	23	62.3	170.6	15.2	23.1	20.2	11.8	280
3	23	47.9	157.5	7.5	11.1	9.5	6.5	230
4	25	67.7	176.4	15.2	15.8	9.1	10.1	280
5	23	66.4	167.6	9.0	14.8	12.5	7.0	285
6	28	59.9	165.1	12.5	22.4	15.6	15.5	285

WT = weight
HT = height

Skinfolds: BI = biceps
TRI = triceps
SUBSCAP = subscapular
SUPRAIL = suprailiac
MAC = mid arm circumference

From these results, % body fat and hence lean body mass can be calculated using the equations of Durnin (section 3.9). Also calculated is body mass index (wt/ht^2). These results are shown in Table 6.4.

Table 6.4 Calculated Anthropometrical Data for the Six Adult Females.

	Body Mass Index	% Body Fat	Total Body Fat (kg)	Lean Body Mass (kg)
1	23.09	35.11	22.61	41.79
2	21.31	32.87	20.48	41.82
3	19.19	22.78	10.91	37.00
4	21.86	27.96	18.93	48.77
5	23.53	25.84	17.16	49.24
6	22.00	31.94	19.13	40.77

The subjects basal metabolic rate was measured using indirect calorimetry.

Table 6.5 Basal Metabolic Rate data for the Six Adult Females.

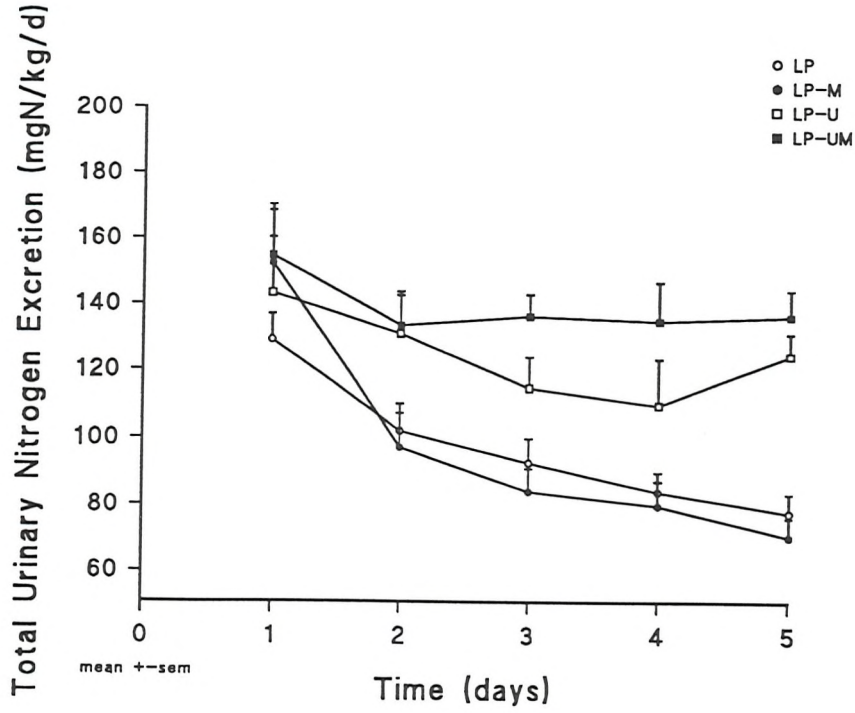
	$\dot{V}O_2$ ml/min	$\dot{V}CO_2$ ml/min	RQ	REE kJ/day	E REQ kJ/day
1	194	187	0.97	5720	8008
2	184	166	0.90	5340	7476
3	161	150	0.94	4700	6580
4	201	176	0.88	5820	8148
5	230	217	0.94	5790	8106
6	201	183	0.91	5850	8190

REE = resting energy expenditure
E REQ = BMR x 1.4

6.3.2 Urinary Nitrogen Excretion

The changes in nitrogen intake were reflected in the urinary excretion of nitrogen (Figure 6.1). Individual data is shown in appendix 4A. On both the 26g protein/day diet (LP) and the 26g protein/day diet with methionine (LP-M), urinary excretion fell during the study period and there was no significant difference between excretion on these two protocols. As with the previous study on males, when urea was supplemented, nitrogen excretion in the urine was increased significantly, with the LP-UM diet resulting in a slightly higher urinary nitrogen loss than the LP-U intake.

Figure 6.1 Urinary nitrogen excretion over the study period in six adult females fed diets of varied sulphur amino acid and nitrogen content.



Urea excretion followed the same patterns, but with the LP-UM diets resulting in a lower excretion of urea than the LP-U diet. The urinary excretion of nitrogen, urea and ammonia over the five day study period are shown in Table 6.6 (appendix 4b and 4C).

Table 6.6 Urinary excretion of nitrogen, urea and ammonia in six adult females over a five day period when consuming diets of varied sulphur amino acid and nitrogen content (mean ±sem).

INTAKE / EXCRETION mgN/kg/d	LP	LP-M	LP-U	LP-UM
NITROGEN				
DAY 1	129 ±19	152 ±18	143 ±17	155 ±14
DAY 2	102 ± 8	97 ±10	130 ±13	133 ± 9
DAY 3	93 ± 7	84 ± 7	115 ± 9	136 ± 7
DAY 4	84 ± 6	80 ± 7	110 ±14	135 ±12
DAY 5	78 ± 6	71 ± 6	125 ± 7	136 ± 8
UREA				
DAY 1	131 ±12	143 ±21	150 ±19	142 ± 9
DAY 2	85 ±12	81 ±10	135 ±13	130 ±10
DAY 3	85 ± 6	71 ± 7	129 ±10	127 ± 8
DAY 4	74 ± 5	71 ±10	111 ±15	126 ±14
DAY 5	61 ± 5	67 ±11	116 ±11	102 ± 7
AMMONIA				
DAY 1	6.1 ±0.6	8.0 ±0.6	6.8 ±0.8	6.3±0.6
DAY 2	4.8 ±0.3	6.3 ±0.7	5.9 ±0.4	5.9±0.5
DAY 3	4.3 ±0.4	5.8 ±0.4	5.1 ±0.3	5.3±0.6
DAY 4	4.2 ±0.5	5.1 ±0.4	4.5 ±0.9	4.9±0.6
DAY 5	4.6 ±0.5	5.8 ±0.7	5.0 ±0.4	4.6±0.5

6.3.3 Apparent Nitrogen Balance

Apparent nitrogen balance was estimated by calculating the difference between nitrogen intake and loss. Nitrogen losses were taken to be total nitrogen excretion in the urine and an amount taken to be 24mg/kg/d for faecal and endogenous losses (Table 6.7 and appendix 4D).

On both the 26g protein (LP) diet and the low protein diet supplemented only with methionine (LP-M), subjects were in negative nitrogen balance, indicating that the diet was deficient in nitrogen. The addition of sulphur amino acids as methionine resulted in an improved apparent balance but this was not significant. Addition of nitrogen as urea to the LP diet (LP-U) improved nitrogen status significantly but subjects were still in negative balance. When urea was supplemented in conjunction with methionine (LP-UM), this beneficial effect of urea was abolished and the apparent nitrogen balance was similar to that seen on the LP diet.

Table 6.7 Apparent nitrogen balance in six adult females consuming diets with different nitrogen and sulphur amino acid content (mean ±sem).

mgN/kg/d	LP	LP-M	LP-U	LP-UM
INTAKE	69 ± 4	70 ± 4	121 ± 7	122 ± 7
URINE	81 ± 5	76 ± 6	116 ± 8	136 ±10
FAECAL & OTHER LOSSES	24	24	24	24
BALANCE	-37^{a} ± 3	-30^{a} ± 4	-19^{b} ± 4	-37^{a} ± 4

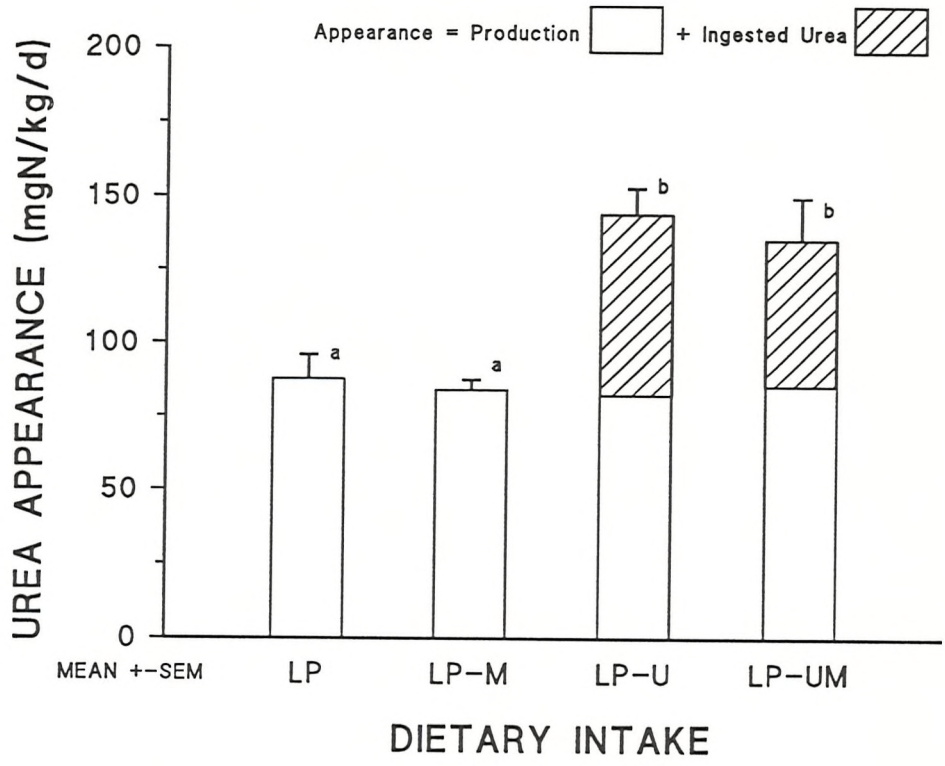
different superscripts indicate significant differences, $p<0.05$.

6.3.4 Urea Kinetics

Plateau enrichment of the doubly labelled urea isotope in urinary urea was identified by visual inspection and the average coefficient of variation at plateau was 16% for [$^{15}N^{15}N$] urea and 17% for [$^{15}N^{14}N$] urea.

Urea appearance (Au), measured by the dilution of [$^{15}N^{15}N$] urea in the urea pool, was 88 $\pm$8 mgN/kg/d on the 26g protein (LP) diet and did not differ when 600mg of methionine was supplemented, having a value of 87 $\pm$4 mgN/kg/d. Supplementation of exogenous urea increased urea appearance in the body pool significantly to 144 $\pm$9 mgN/kg/d on the LP-U diet and 135 $\pm$14 mgN/kg/d on the LP-UM diet. When the amount of urea ingested (Iu) was subtracted from the appearance in the urea supplemented diets, giving the endogenous production (Pu), it was found to be similar to that of the first two dietary regimens (ie. 91 $\pm$9 and 82 $\pm$13 mgN/kg/d respectively). The increase in appearance was entirely due to ingested urea and endogenous production was not significantly different in the four study periods. This is clearly shown in Figure 6.2 (and appendix 4E) and is the same finding as for the previous chapter.

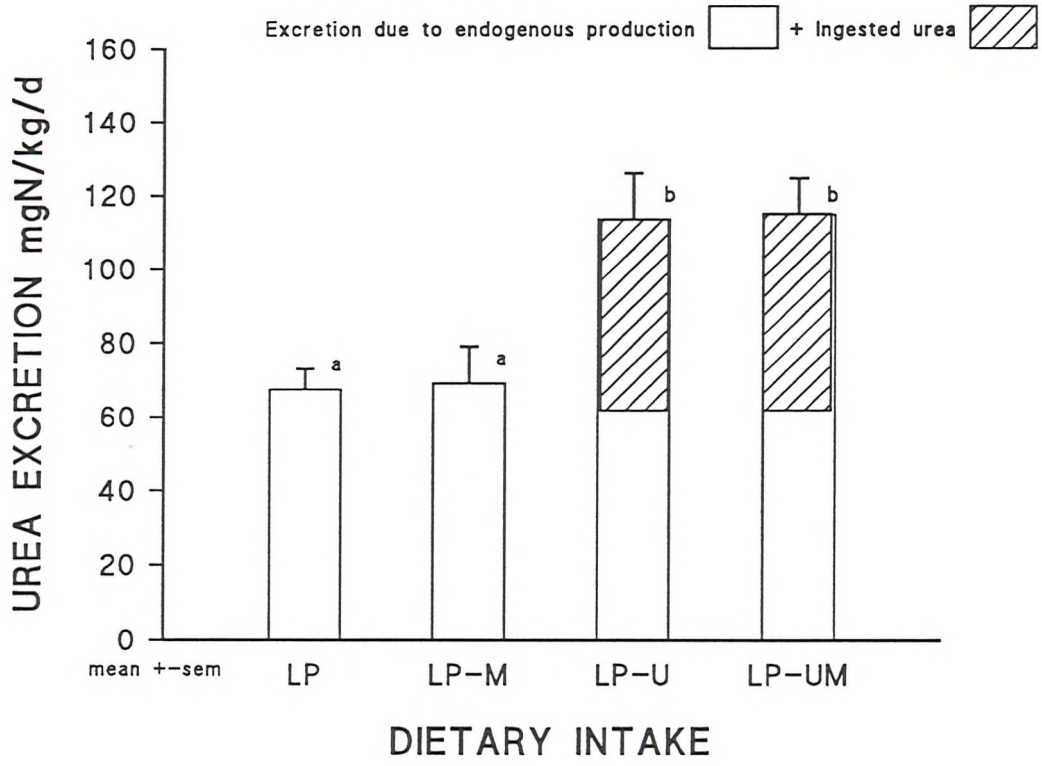
Figure 6.2 Appearance of urea in the body pool for six adult females fed diets with varying nitrogen and sulphur amino acid intakes.



Individual results for urinary urea excretion are shown in appendix 4B. On an intake of 26g protein/day (LP diet), urea excretion amounted to 68 ±6 mgN/kg/d. When methionine was added to this basic diet, excretion of urea was not significantly different, being 69 ±10 mgN/kg/d. When exogenous urea was ingested, urinary urea excretion rose significantly to 113 ±13 mgN/kg/d on the LP-U diet and 115 ±10 mgN/kg/d when methionine

was also supplemented (LP-UM). When the proportion of urea ingested was taken from the value of excretion on the two urea supplemented diets, the 'real' excretion estimated to be due to endogenous production was found to be similar for all four diet groups but with a trend for the urea supplemented diets to be the lowest.

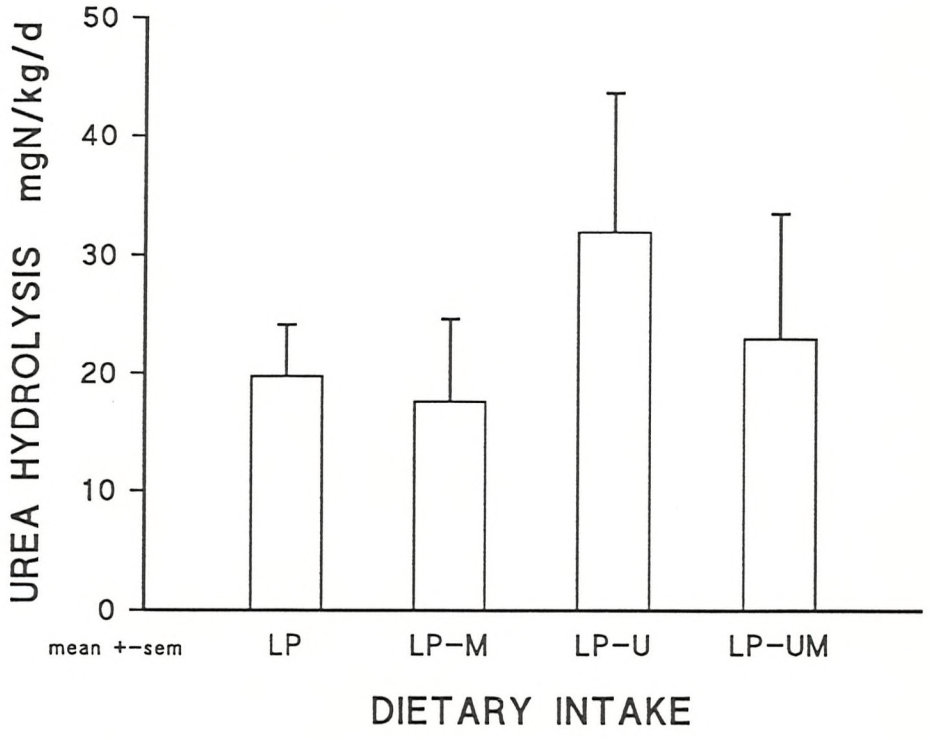
Figure 6.3 Urinary urea excretion in six adult females consuming diets with varied nitrogen and sulphur amino acid content.



Urea excretion represents the proportion of urea leaving the body pool which has not passed to the colon for hydrolysis. Hence, the results for hydrolysis (T) reflect the drop in

'real' excretion with ingested urea by an increase in hydrolysis, although differences did not reach statistical significance (appendix 4F). On the basic 26g protein diet, colonic hydrolysis was 20 ±4 mgN/kg/d. With a 600mg methionine supplement (LP-M), hydrolysis did not alter, with a rate of 18 ±7 mgN/kg/d. When urea was introduced to the pool from ingestion on its own (LP-U), hydrolysis in the colon roses to 32 ±12 mgN/kg/d, although this increase is not significant due to a wide inter-individual variability in response. When this urea supplement is in conjunction with methionine (LP-UM), hydrolysis occurred at a rate of 23 ±11 mgN/kg/d.

Figure 6.4 Urea hydrolysis in six adult females consuming diets of varied nitrogen and sulphur amino acid content.



The relative fates of nitrogen returned to the body after hydrolysis are given in Table 6.8 and appendix 4G. The majority of nitrogen was reincorporated into the body via synthetic pathways (S), with only 15% passing back to urea production (Pr) and measured as $^{15}N^{14}N$ in urine. Retention was greater on an intake with exogenous urea only (LP-U), reflecting the improved nitrogen balance; the addition of methionine (LP-UM) returning the values close to that of the LP diet.

Table 6.8 Urea kinetics for six adult females consuming diets with varied nitrogen and sulphur amino acid contents (mean ±sem).

mgN/kg/d	LP	LP-M	LP-U	LP-UM
Intake	69^{a} ± 4	70^{a} ± 4	121^{b} ± 7	122^{b} ± 7
Appearance	88^{a} ± 8	84^{a} ± 4	144^{b} ± 9	135^{b} ±14
Production	88 ± 8	84 ± 4	91 ± 9	82 ±13
Excretion	68^{a} ± 6	69^{a} ±10	113^{b} ±13	115^{b} ±10
Hydrolysis	20 ± 4	18 ± 7	32 ±12	23 ±11
Urea Formation(Pr)	2.7^{a}±0.4	2.2^{a}±0.2	5.0^{b}±0.8	4.0^{ab}±0.7
Synthetic Pathways(S)	17 ± 4	16 ± 7	28 ±11	20 ±10

different superscripts indicate significant differences, $p<0.05$. Superscript ^{ab} significantly different to LP-M.

6.3.5 Plasma Urea

Plasma urea concentration was measured and found to be 17.3 ±2 mgN/100ml on an protein intake of 26g/day. This was not significantly different when methionine was added to the diet, being 16.4 ±2 mgN/100ml. Surprisingly, the urea pool was not expanded when 6.9g of urea was ingested daily on the LP-U diet, reflected in a plasma urea concentration of 13.7 ±3 mgN/100ml. However, when methionine and urea were supplemented together (LP-UM), an expansion of the urea pool was seen and plasma urea level rose to 28.1 ±2 mgN/kg/d. Results for plasma urea and urea pool size are shown in Table 6.9 (and appendix 4H). It was also shown that urea excretion and hydrolysis are not related to plasma urea levels (correlations r=0.435 and r=0.421 respectively) as previously discussed in chapter 5.

Table 6.9 Plasma urea levels and urea pool size in six adult females consuming diets with varied nitrogen and sulphur amino acid content (mean ±sem).

	LP	LP-M	LP-U	LP-UM
Plasma Urea mgN/100ml	17.3^{a} ± 2	16.4^{a} ± 2	13.7^{a} ± 3	28.1^{b} ± 2
Urea Pool grams	5.3^{a} ±0.5	5.1^{a} ±0.6	4.2^{a} ±0.8	8.7^{b} ±0.8

different superscripts indicate significant differences, $p<0.05$.

6.3.6 Urinary Excretion of Inorganic Sulphate

In contrast to the other data, which are a reflection of the nitrogen content of the diet, sulphur excretion, measured by inorganic sulphate levels in urine, was determined by the sulphur amino acid content in the diets. On the 26g protein diet alone (LP), inorganic sulphate excretion in the urine was 14.5 ±2 mmol/d, and did not differ significantly to excretion on the LP-U diet. With addition of dietary sulphur as methionine (LP-M and LP-UM), inorganic sulphate excretion rose significantly (see appendix 6 for individual results).

Table 6.10 Inorganic sulphate excretion in six adult females consuming diets with varied nitrogen and sulphur amino acid content (mean ±sem).

	LP	LP-M	LP-U	LP-UM
Inorganic Sulphate Excretion (mmol/d)	14.5^{a} ±2.0	20.6^{b} ±2.0	14.0^{a} ±0.3	19.1^{b} ±0.8

different superscripts indicate significant differences, $p<0.05$.

6.4 Discussion

This study was designed to examine if sulphur amino acids were limiting the colonic nitrogen salvage system when protein intake was low. It was demonstrated that addition of methionine to a low protein intake did not improve nitrogen status and hence it is unlikely to be the first limiting component under these conditions. In comparison to the male study described in chapter 5, women were able to utilise the smaller supplement of urea by increasing urea hydrolysis in the colon to improve nitrogen balance.

The study was constructed in order to determine whether sulphur amino acids were contributing to the 'switching off' of urea salvage on protein intakes less than 35g/day. Women were used instead of men, due to subject availability and the fact that the majority of our understanding of nitrogen metabolism has been gained from young male subjects so more measurements are required in women. However, using women meant controlling for the cyclic changes throughout the menstrual cycle. All subjects were taking the oral contraceptive pill which allowed for increased confidence in the timing of the measurement. Every study was carried out during week 4 (with week 1 taken as the commencement of menstruation) in each subject in order to control for known differences in urea kinetics between ovulation and the luteal phase (McClelland 1994).

The study protocol followed was as for the previous male study, except that the low protein intake was lowered to 26g rather than 30g protein/day. This meant that the intake was constant at 0.42g/kg/d for both groups of subjects. There is no evidence as to the lower limit of protein intake for urea kinetics in women and whether this is the same as for the 35g/day seen in men. However, the failure of the women in this study to reach nitrogen equilibrium on the LP diet, would suggest that this intake of 26g protein/day is below the minimal requirement level.

The sulphur amino acids, in the form of methionine, were given at a level of 600mg to make the content of the diet up to the current recommendation of 900mg/day and this provided only 0.06gN, so any effect would be due to the sulphur, not the nitrogen content of the supplement. This was given as a blackcurrant drink in order to disguise the strong flavour of the sulphur amino acid. L-methionine was used as this is more effective than the other isomers of the amino acid (Kies & Fox 1975, Zezulka & Calloway 1976b). Energy intake was similar to the male study when considered in terms of intake per kilogram body weight and was calculated to satisfy BMR x1.4 as recommended for a sedentary lifestyle. Non-essential nitrogen, as urea, was supplemented at the lower level seen in the previous study ie. 6.9g urea (3.2gN), in order to see if the sulphur amino acids could influence the bacterial utilisation of this nitrogen source and was added to the blackcurrant drink alone or along with the methionine. However, the results clearly show that the women were able to utilise the urea supplement before methionine was added so any beneficial influence may have been masked.

One conclusion reached in this study is that men and women differ in their handling of a 6.9g urea supplement on a low protein diet. Unlike the men, these female subjects were able to improve their nitrogen balance by utilising the urea-nitrogen via colonic recycling. There is no evidence to suggest gender differences in the numbers or types of colonic microflora but differences are clear in urea kinetics. Rates for endogenous urea production are lower per kilogram body weight in women, a result also seen in another study carried out in our group (Child 1995). Hence, one contributing factor to the extent of urea salvage may be body composition (cf general discussion).

The other factor which differed between men and women was the protein content of the low protein diet. Although similar per kilogram body weight (0.42g/kg/d), it is not known where the 'switch point' for failure of urea salvage to maintain nitrogen

balance lies in women. Hence 26g of protein may be closer to the switch point in women than the 30g protein in men, allowing the smaller urea supplement of 6.9g to be utilised by the colonic bacteria. It was again clear that urea hydrolysis was not a concentration dependent process as there was an increase in hydrolysis despite no enlargement in the urea pool with the LP-U diet.

The main purpose of this series of feeding experiments was to look at the influence of sulphur amino acids on the colonic salvage system. Past studies have been unclear as to the beneficial effect of methionine on low protein diets. Early animal experiments showed that additional methionine increased nitrogen retention in dogs and rats (Cox *et al* 1947, Eckert 1948). Much of the research carried out with supplemental methionine has involved adding methionine to important protein sources which are limiting in this amino acid, such as potatoes, soybeans and other legumes. Human studies by Kies and fellow workers demonstrated the beneficial effects on nitrogen balance of additional methionine added to diets at low levels of nitrogen intake, but at higher nitrogen intakes the effects were less clear (Kies & Fox 1972b, Kies 1975, 1978, Zezulka & Calloway 1976). A similar influence of nitrogen intake was again shown by Young who observed that methionine had a supplementary effect on soy protein isolate only in subjects given a low protein diet with an adequate concentration of added methionine (Young *et al* 1984). Suggestions as to the underlying mechanism behind this effect range from an increase in whole body protein synthesis (Muramatsu *et al* 1986) to an influence on lipid metabolism (Roth & Allison 1949, Smith *et al* 1983). Methionine has also been found to improve growth of infants fed soy based formulas (Fomon *et al* 1979). However, this effect has been difficult to replicate (Koishi *et al* 1984, Kaneko *et al* 1986).

This study was unable to demonstrate any beneficial effects of methionine on nitrogen balance at nitrogen intakes of this level. There may be several reasons for this observation. The

first is that the first limiting factor, non-essential nitrogen does not appear to be limiting in this case, as shown by the ability of these women to utilise the small urea source. This may influence any effects seen with supplemental methionine.

Another important consideration is that the requirement level for methionine may not have been reached, either due to an incorrect assessment of the dietary methionine content by the comp-eat computer analysis, which could have limitations when applied to the actual dietary intake, or that the requirement level is not as recommended, ie 900mg/day. Requirement levels for methionine have been investigated on numerous occasions with varied results ranging from 260mg to 2.0g/day (Rose 1955, Swendseid *et al* 1956, Reynolds *et al* 1958, Clarke *et al* 1966, Young *et al* 1991). The main problem is that the requirement level is influenced by many other factors such as total nitrogen intake, nitrogen source, relative abundance of cysteine and glycine, and even amounts of methyl groups present (Young *et al* 1991). A very important factor in this diet was the abundance of cysteine. Dietary methionine and cysteine requirements are often grouped together to give a total sulphur amino acid requirement because of the sparing effect of cysteine on methionine requirements (Finkelstein & Mudd 1967). The LP diet contained 280mg cysteine which has been demonstrated to reduce the requirement for methionine to as little as 260mg (Clarke *et al* 1970). Hence, 900mg/day is an estimated recommendation and may well not be the requirement level for the females in this study.

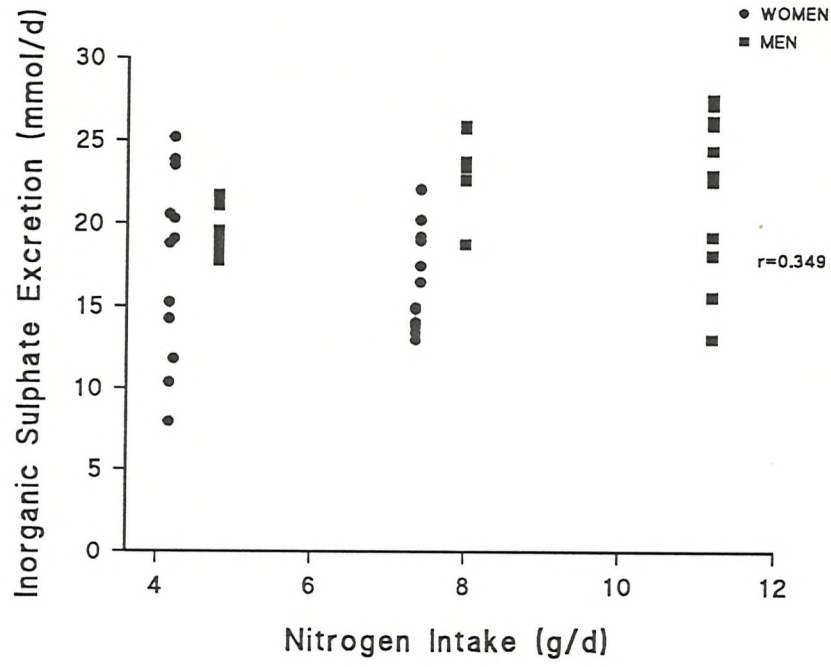
More importantly, if the requirement for methionine has been exceeded then this will have a negative influence on nitrogen balance. That there is a maximal level to the beneficial effects of methionine supplementation was shown in a number of rats studies, with excess methionine reducing growth and demonstrating toxicity (Brown & Allison 1948, Wretland & Rose 1950, Cohen *et al* 1958, Klain *et al* 1963). In the human studies described earlier, it too was found that supplements above 1.1g methionine/day to diets containing approximately 4.0gN/day abolished any improvements in the subjects nitrogen balance

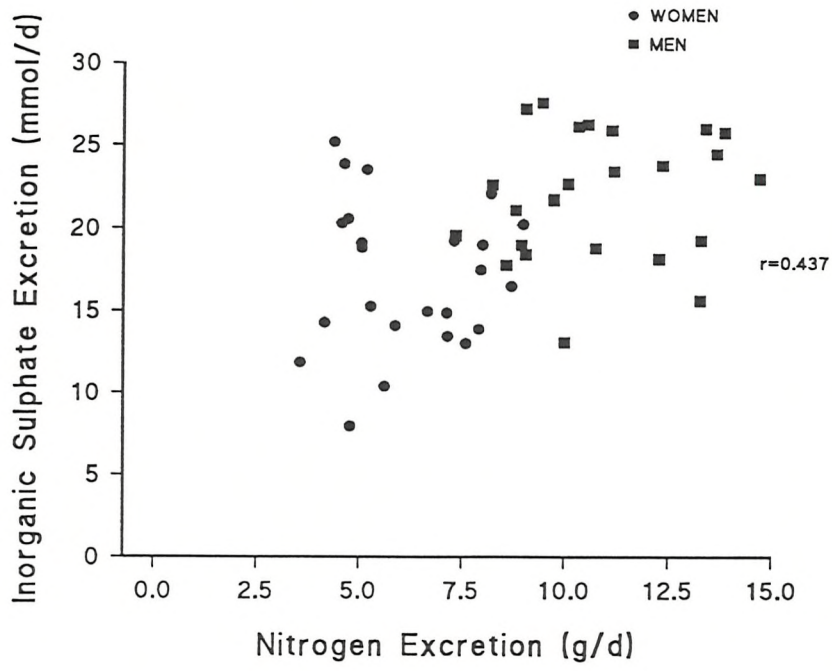
seen on lower additions (Kies & Fox 1975, Young *et al* 1984). So any error in estimation of the level of supplementation used here, ie. 600mg/day which brought levels of this sulphur amino acid to high levels would in fact be detrimental to the nitrogen balance of the subjects. The toxicity seen with excess methionine can be alleviated with supplementary glycine (Roth & Allison 1949, Benevenga & Harper 1967) and the links of the urea salvage system with this amino acid will be looked at in the next chapter.

A final hypothesis as to why the urea salvage system failed to increase nitrogen recycling with supplemental methionine is that on these low levels of nitrogen intake, there were insufficient resources with which to handle the sulphur amino acids, either by the body or the bacteria themselves. Faecal sulphate excretion remains constant despite sulphur supplementation (Eckert 1948, Wright *et al* 1960), a result that suggests that all protein sulphur is absorbed. This means that the methionine supplement may not reach the colonic microflora and the beneficial effects seen in some studies could be occurring on the whole body level, rather than simply an influence on the metabolism of colonic bacteria.

So how do supplemental sulphur amino acids influence the body's nitrogen balance? In many cases, it appears that there is a parallelism between nitrogen and sulphur balances. They occur together in the proteins of food and the body and in general, excretion of nitrogen follows the same pattern as urinary sulphate excretion (Eckert 1948, Wright *et al* 1960, Scrimshaw *et al* 1969). However, it has also been demonstrated that this relationship fails when low protein diets limiting in the sulphur amino acids are fed, with or without methionine supplementation (Bressani *et al* 1965, Clarke *et al* 1970), or when calories are limiting (Bressani *et al* 1965, Jourdan *et al* 1980).

Figure 6.5 The relationship between urinary sulphate excretion and nitrogen excretion and nitrogen intake in six adult females consuming diets with varying nitrogen and sulphur amino acid content.





1980). The results from this study agree with this statement in that sulphur excretion (in the form of urinary inorganic sulphate excretion), is closely related to sulphur intake but did not demonstrate a relationship with nitrogen excretion, possibly due to the non-essential nitrogen manipulations. Previous studies have demonstrated that methionine supplementation increased nitrogen retention in the body (Eckert 1948, Bressani *et al* 1965) but the present studies were unable to demonstrate this. The results were also in agreement with observations that show methionine supplementation resulted in increased inorganic sulphate excretion, as all of the supplemental methionine is absorbed and excreted in the form of inorganic sulphate (Johnson *et al* 1947, Eckert 1948). The urinary sulphate excretion was plotted against nitrogen intake in Figure 6.5 for both the male and female studies. This does not demonstrate any linear relationship between the two.

In conclusion then, sulphur amino acids, as methionine were not shown to improve nitrogen balance or enhance the urea salvage system on protein intakes at this level. This may have been due to the inability of the colonic bacteria to utilise the sulphur amino acids or because the level of methionine exceeded requirement levels. Excess methionine intake has been shown to be detrimental to nitrogen status and can lead to toxic effects if levels are increased further. Hence, it appears that under these conditions sulphur amino acids are not a limiting factor but this may have been masked by the fact that, in the women, non-essential nitrogen was the primary limiting factor at this level as they were able to utilise the smaller urea supplement of 6.9g urea. In the male subjects, other limiting factors were influential but these conditions were not replicated in the women at an intake of 26g protein.

6.5 Summary

The present study demonstrates that on this margin of protein intake, women were able to utilise a small amount of urea nitrogen by recycling in the colon to improve nitrogen balance. In comparison, male subjects in the previous study (chapter 5) were unable to utilise this level of non-essential nitrogen. However, the addition of methionine to this diet did not improve nitrogen balance. This may have been due to the nitrogen intake level, an inability of the colonic bacteria to handle the load of sulphur amino acids or an excess of methionine. It is therefore unlikely that methionine plays a limiting role in the salvage of urea nitrogen under these conditions.

CHAPTER 7

THE INFLUENCE OF GLYCINE ON UREA-NITROGEN SALVAGE

It has been demonstrated that the primary influence on urea-nitrogen salvage is the intake of total nitrogen. The importance of non-essential nitrogen was illustrated by its ability to enhance urea hydrolysis and improve nitrogen balance when protein intake was restricted. This chapter will investigate whether the source of non-essential nitrogen is influential on the salvage system and look more closely at the role of non-essential amino acids, particularly glycine.

7.1 Introduction

Attention has been focused on the amino acid glycine because of its central position in mammalian metabolism, with close ties to the metabolism of serine and the sulphur amino acids. As well as being a constituent of body proteins, especially collagen, glycine acts as a precursor for a range of important metabolites, for example conjugates such as glycocholic acid, purines, haem, creatinine and glutathione. Each of these represents a significant daily drain on the availability of glycine as they are end products with no return to the metabolic pool (Jackson 1991b).

This considerable demand for glycine is further exacerbated under certain conditions when it becomes limiting, leading to its classification as a conditionally essential amino acid (Jackson 1983). These conditions occur at crucial stages in an individuals life for nutrition when there is an extra demand for glycine for rapid growth, such as pregnancy (Persaud *et al* 1989), recovery from malnutrition especially in children, and in the preterm infant (Jackson *et al* 1981b).

Thus the need for the synthesis of substantial amounts of non-essential amino acids is apparent. In the past, nitrogen balance studies have demonstrated that one approach is to

supplement non-essential amino acids in the diet when protein is restricted to improve nitrogen balance. In particular, Snyderman demonstrated that by adding glycine to a low protein diets, infants regained weight and maintained nitrogen balance (Snyderman *et al* 1962). When the source of this non-essential nitrogen was examined, it was found that glycine was more efficient at improving nitrogen retention in adults than diammonium citrate or urea, improving even further when glycine was supplemented with diammonium citrate or glutamate (Swendseid *et al* 1960, Clarke *et al* 1973, Anderson *et al* 1970, Kies 1974). Recent stable isotope studies, have demonstrated that in normal adults on a low amino acid diet (0.6g protein/kg/d), glycine production in the body was insufficient to satisfy the normal metabolic demands (Yu *et al* 1985).

Because of the essentiality of glycine under conditions of limited protein intake or increased protein demands, it is logical that the adaptive role of urea salvage may be important in contributing to the satisfaction of these needs. Hence, the aim of the series of feeding experiments described in this chapter was to investigate whether glycine is a limiting factor to urea nitrogen hydrolysis during low protein intake. Also the relative effectiveness of glycine and urea as non-essential nitrogen sources were compared using the technique of urea kinetics. Further, the influence of the experimental low protein diets with the varying supplements on glycine metabolism was looked into using urinary 5-oxoproline as a marker of glycine insufficiency (Jackson *et al* 1987).

7.2 Protocol and Methods

7.2.1 Protocol

The studies were carried out in the same six adult females aged 22-28 as featured in the preceding chapter. The subjects agreed to participate after the study had been explained to them. They were all in good health at the time of the studies. The study had the approval of the Southampton Hospitals and South West Hampshire Health Authority Ethical Subcommittee. Each subject underwent an additional protocol to the four described in the previous chapter, three will be discussed in this chapter (Table 7.1). Each study period lasted for five days over which subjects were given a standard diet. Basal metabolic rate measurements and anthropometrical data were as described previously in section 6.3.1. Complete collections of urine were made for the duration of the study, and during the last 24 hours of the study period measurements of urea kinetics were made. A plasma sample was taken at 3pm on day 5 and stored at -70^{0}C. The studies were carried out in the order shown and at the same stage in the menstrual cycle (week 4, the luteal phase) and hence, three weeks passed between consecutive studies.

Table 7.1 The Dietary Protocol

DIET	PROTEIN	UREA	GLYCINE	TOTAL NITROGEN
LP	26g	---	---	4.16g
LP-U	26g	6.9g	---	7.36g
LP-UG	26g	3.4g	8.6g	7.36g

7.2.2 Diets

The low protein diet (LP) was fed at a level of 26g protein/day as in the previous chapter. The diet was made of whole food as shown in Table 6.2 and appendix 1C. The urea dose for the LP-U diet was 6.9g/d (3.2gN/d), the smaller dose of the two used in Chapter 5. L-glycine was supplemented along with urea on the LP-UG diet so that there was a source of urea for the colonic bacteria to utilise. The supplement was calculated to contain the same amount of nitrogen as the urea supplement (3.2gN/d), but having 1.6gN as urea and 1.6gN as glycine. The supplements were given as a blackcurrant drink with meals. Food was eaten ad libitum on days 1-4, but on day 5, food was eaten at three hourly intervals to coincide with isotope administration.

7.2.3 Urea Kinetics

The protocol used the prime intermittent dose method of urea kinetics (see section 3.1). During the final 24 hours of each study period, urea kinetics was measured as described in section 6.2.3. An accurately measured prime dose of approx. 28.5mg was given orally at midnight on day 5 and then starting at 6am, intermittent doses of 5.5mg were given at three hourly intervals until 6pm.

7.2.4 Urine Collection

Total urine collection occurred throughout the five day protocol period as described in section 6.2.4. For days 1-4 urine was collected as twelve hour collections. After isotope administration, urine was collected at three hourly interval sin order to coincide with isotope administration. Urine was collected into plastic containers containing 2ml 6M HCl/100ml volume to act as a preservative.

7.2.5 Analyses

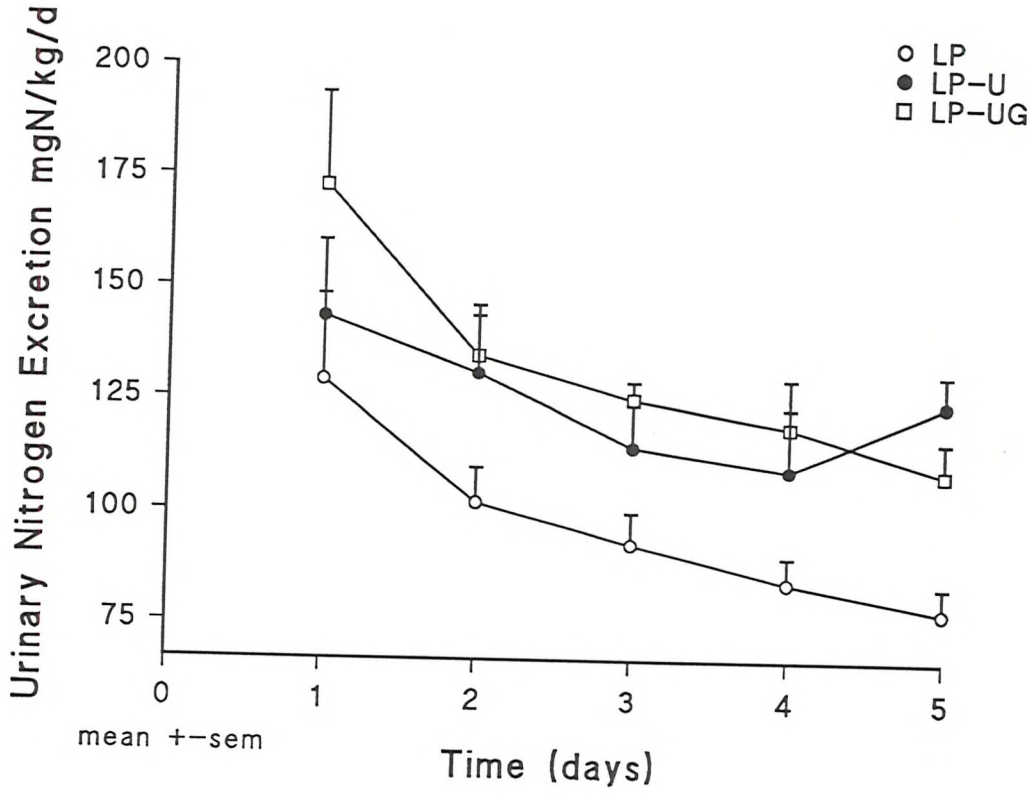
The nitrogen content of the urine was measured by Kjeldahl analysis (section 3.3). The concentration of urea and ammonia in urine and plasma urea were determined by the Berthelot method (section 3.2). Urea nitrogen was isolated from urine for mass spectrometry using short ion-exchange column chromatography (section 3.4) and nitrogen gas liberated from this urea by reaction with alkaline hypobromite (section 3.5). The relative proportions of $^{15}N^{15}N$ urea, $^{15}N^{14}N$ urea and $^{14}N^{14}N$ urea in this nitrogen gas were determined using a triple collector isotope ratio mass spectrometer. Inorganic sulphate was measured in the urine (section 3.7). Urinary levels of 5-oxoproline were measured in samples from both the male and female feeding experiments by Dr C. Persaud (see section 3.8).

7.3 Results

7.3.1 Urinary Nitrogen Excretion

Changes in the dietary content of nitrogen were reflected in the loss of nitrogen in the urine. On the LP diet, nitrogen excretion fell from day one to a lower level on day 5 by which time excretion was indicating that the body was coming into a new metabolic steady state. Excretion of nitrogen was higher on the diets supplemented with urea (LP-U) or urea and glycine (LP-UG), and losses on these two intakes were not significantly different to each other.

Figure 7.1 Urinary nitrogen excretion over the five day study period in six adult females consuming diets with varying nitrogen source.



Urea excretion over the five day period followed the same pattern as nitrogen excretion, as the majority of nitrogen excreted in the urine is urea. The LP diet resulted in lower urinary urea excretion than the urea supplemented diets. The urea supplement with glycine ie. LP-UG, did tend to show a lower urea excretion than the LP-U diet but this did not reach significance. There were no differences in the excretion of ammonia, which seems to suggest that alterations in urea are the main contribution to changes in total nitrogen excretion. Relative excretions of nitrogen, urea and ammonia are shown in Table 7.2 and appendices 5A, 5B and 5C.

Table 7.2 Urinary excretion of nitrogen, urea and ammonia in six adult females over a five day study period when consuming diets of varied nitrogen source (mean ±sem).

INTAKE EXCRETION mgN/kg/D	LP	LP-U	LP-UG
NITROGEN			
DAY 1	129 ±19	143 ±17	173 ±21
DAY 2	102 ± 8	130 ±13	135 ±11
DAY 3	93 ± 7	115 ± 9	125 ±10
DAY 4	84 ± 6	110 ±14	119 ±11
DAY 5	78 ± 6	125 ± 7	109 ± 7
UREA			
DAY 1	131 ±12	150 ±19	167 ±33
DAY 2	85 ±12	135 ±13	113 ± 7
DAY 3	85 ± 6	129 ±10	115 ±15
DAY 4	74 ± 5	111 ±15	108 ±13
DAY 5	61 ± 5	116 ±11	106 ± 7
AMMONIA			
DAY 1	6.1 ±0.6	6.8 ±0.8	8.1 ±1.7
DAY 2	4.8 ±0.3	5.9 ±0.4	5.7 ±0.4
DAY 3	4.3 ±0.4	5.1 ±0.3	5.1 ±0.4
DAY 4	4.2 ±0.5	4.5 ±0.9	5.3 ±0.5
DAY 5	4.6 ±0.5	5.0 ±0.4	5.5 ±0.5

7.3.2 Apparent Nitrogen Balance

Apparent nitrogen balance was estimated in the same way as before by calculating the difference between nitrogen intake and loss. Nitrogen losses were taken to be total nitrogen excretion in the urine (a value per 24 hours taken over days 4 and 5) and an amount taken to be 24mg/kg/d for faecal and other endogenous losses (Table 7.3 and appendix 5D).

As seen in the previous chapter, the 26g protein/day LP diet was clearly insufficient as all subjects had a large deficit in nitrogen intake compared to excretion. Addition of the urea supplement (LP-U), increased nitrogen balance significantly but subject were still in overall negative balance. There was no difference between this result and nitrogen balance when the non-essential nitrogen supplement was partly glycine (LP-UG).

Table 7.3 Apparent nitrogen balance in six adult females consuming diets with varying nitrogen sources (mean ±sem).

mgN/kg/d	LP	LP-U	LP-UG
INTAKE	69 ± 4	121 ± 7	121 ± 7
URINE	81 ± 5	116 ± 8	114 ± 9
FAECAL AND OTHER LOSSES	24	24	24
BALANCE	-37^{a} ± 3	-19^{b} ± 4	-17^{b} ± 5

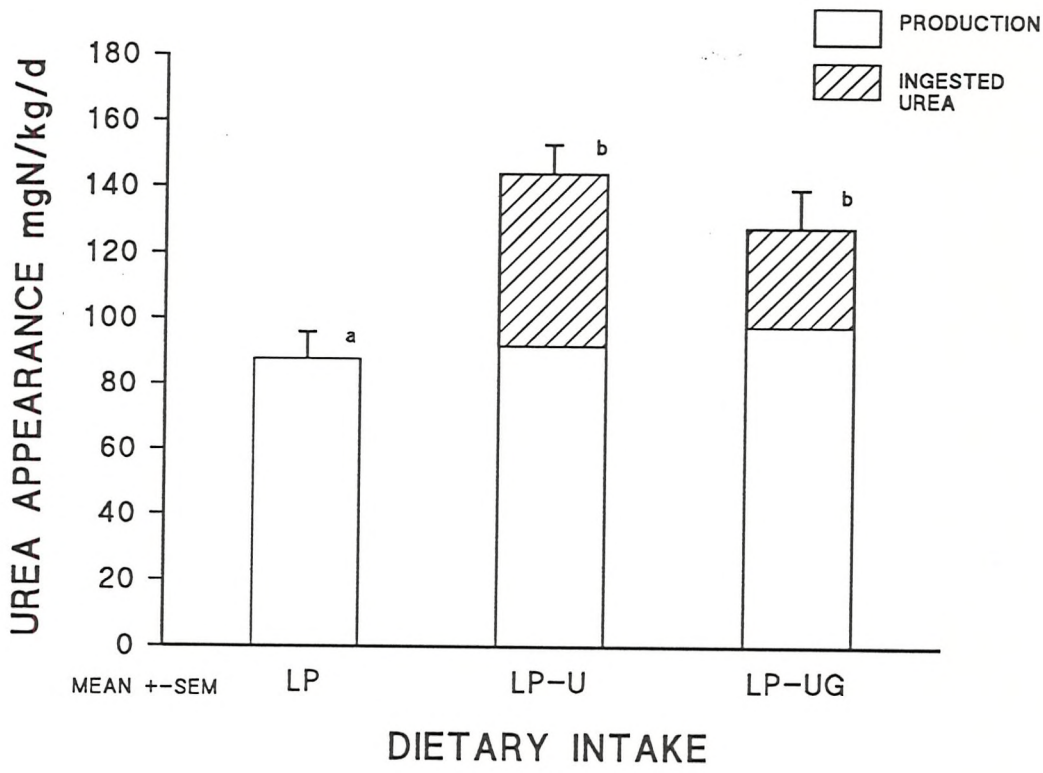
different superscripts indicate significant differences, $p<0.05$.

7.3.3 Urea Kinetics

Urea appearance was measured by the dilution of the doubly labelled isotope to unlabelled urea in the body pool and hence, reflected in urinary excretion of the relative enrichments. Plateau of enrichment for the calculation of urea kinetics was identified by visual inspection of a plot of enrichment over time. Individual data for the six adult females is shown in appendix 5E.

On the LP intake, appearance of urea in the urea pool occurred at the rate of 88 $\pm$8 mgN/kg/d. When additional urea was added to this protein intake in the form of a supplement (LP-U), appearance rose significantly to 144 $\pm$9 mgN/kg/d. Appearance dropped slightly to 128 $\pm$11 mgN/kg/d when a portion of the supplemental urea was replaced with glycine (LP-UG), but this difference was not statistically significant. Hence, appearance of urea in the body pool reflected the intake of nitrogen in the diet, regardless of source. When the appearance due to the supplemented urea was taken into account (see Figure 7.2), the endogenous production of urea from body and dietary amino acids via the urea cycle was seen to be constant, reflecting the consistent protein intake of all three dietary regimens.

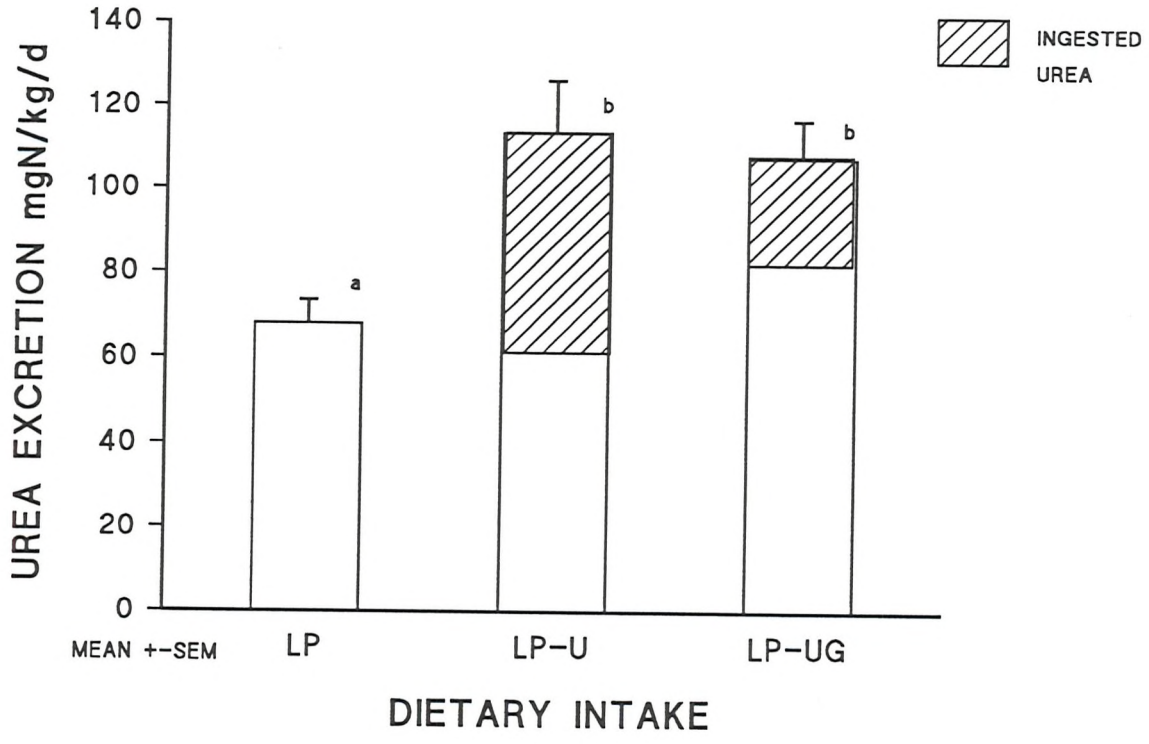
Figure 7.2 Appearance of urea in the body pool in six adult females consuming diets with varied sources of nitrogen.



Urea excretion (shown in Figure 7.3 and appendix 5B) occurred at a rate of 68 ±6 mgN/kg/d on the LP diet. When exogenous urea was included in the dietary intake, urea appearance in the pool was increased and urinary excretion was elevated. On the LP-U intake excretion rose to 113 ±13 mgN/kg/d. When the urea supplement was partly replaced with glycine (LP-UG), excretion fell to 107 ±9 mgN/kg/d, but this fall was not significant.

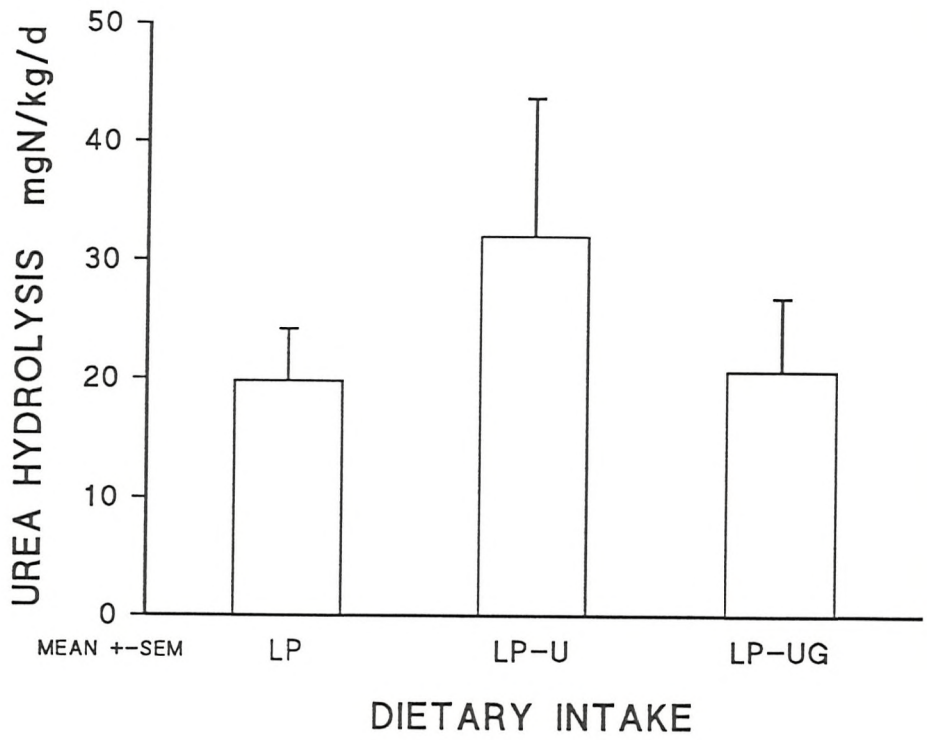
When the excretion due to the ingested urea was taken into account, the estimated values for excretion of endogenously formed urea was 61 ±10 mgN/kg/d on the LP-U intake and 55 ±8 mgN/kg/d for the LP-UG diet. These values were not significantly different to the rate of excretion on the LP diet.

Figure 7.3 Urinary urea excretion of six adult females consuming diets with varied nitrogen source.



Hydrolysis of urea was estimated from the proportion of produced urea nitrogen that was not accounted for by excretion. Results are shown in Figure 7.4 and appendix 5F. On average, 23% of urea production passed to the colon on the LP intake, giving a rate of urea hydrolysis of 20 ±4 mgN/kg/d. When urea was supplemented alone (LP-U) at a rate of 6.9g/d (3.2gN/d), a similar proportion of urea appearance passed to the colon, so that urea hydrolysis rose to 32 ±12 mgN/kg/d. This rise did not ,however, reach statistical significance. When glycine and urea contributed to the non-essential nitrogen source (LP-UG), hydrolysis fell back to 21 ±6 mgN/kg/d, equivalent to on 16% of urea appearance, so a greater proportion was excreted.

Figure 7.4 Urea hydrolysis in six adult females consuming diets of varied nitrogen source.



The relative fates of nitrogen returned to the body by urea salvage are given in Table 7.4 and appendix 5G. The majority of nitrogen was incorporated into the body's nitrogen pool via synthetic pathways (S), leaving only a small proportion (13-23%) which was returned to the urea cycle and excreted as urea in the urine (Pr), measured by the relative excretion of $^{15}N^{14}N$ urea. Retention of urea-nitrogen was greater on the LP-U diet than both the LP and LP-UG intakes, suggesting that this was the most efficient nitrogen source for enhanced urea salvage.

Table 7.4 Urea Kinetics for six adult females consuming diets with varied nitrogen source (mean ±sem).

mgN/kg/d	LP	LP-U	LP-UG
Intake	69^a ± 4	121^b ± 7	121^b ± 7
Appearance	88^a ± 8	144^b ± 9	128^b ±12
Production	88^a ± 8	91^a ± 9	101^a ±10
Excretion	68^a ± 6	113^b ±13	107^b ± 9
Hydrolysis	20 ± 4	32 ±12	21 ± 6
Urea Formation (Pr)	2.7^a ±0.4	5.0^b ±0.8	5.4^b ±0.7
Synthetic Pathways (S)	17 ± 4	28 ±11	16 ± 5

different superscripts indicate significant differences, $p<0.05$.

7.3.4 Plasma Urea

A sample of plasma was taken at 3pm on day 5 and the urea concentration determined and urea pool size calculated (Table 7.5 and appendix 5H). On a 26g protein (LP) intake, plasma urea was found to be at a level of 17.3 ±2 mgN/100ml. When urea was added to the diet in the LP-U supplement, plasma urea did not rise, being at a level of 13.7 ±3 mgN/100ml and hence, the urea pool was not expanded. Hence, this indicated that the extra urea appearing in the pool was not contributing to an expansion in the urea pool size, but was directed through to hydrolysis or excretion. When the supplement consisted of urea and glycine (LP-UG), plasma urea levels rose to 22.1 ±3 mgN/100ml, suggesting the urea was held in an expanded pool.

Table 7.5 Plasma urea and urea pool size in six adult females consuming diets of varied nitrogen source (mean ±sem).

	LP	LP-U	LP-UG
Plasma Urea mgN/100ml	17.3^{a} ± 2	13.7^{a} ± 3	22.1^{b} ± 3
Urea Pool (gm)	5.3^{a} ±0.5	4.2^{a} ±0.8	6.8^{ab}±1.0

different superscripts indicate significant differences, $p<0.05$. ^{ab}-significantly different to LP-U.

7.3.5 Inorganic Sulphate Excretion

The excretion of sulphur in the urine, as reflected by inorganic sulphate excretion (see Table 7.6 and appendix 6), was 14.5 ±2 mmol/d on the 26g protein/day diet. The addition of supplemental nitrogen did not influence this marker, nor did the source of the nitrogen, with no significant changes in inorganic sulphate excretion on either the LP-U or LP-UG.

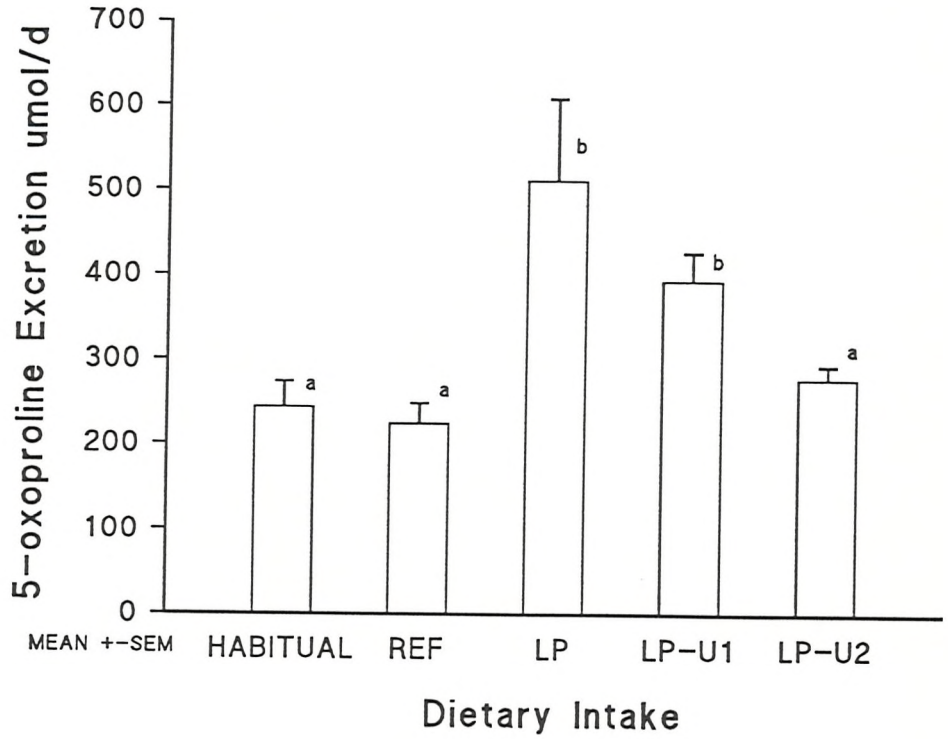
Table 7.6 Urinary excretion of inorganic sulphate in six adult females consuming diets of varied nitrogen source (mean ±sem).

	LP	LP-U	LP-UG
Inorganic sulphate excretion (mmol/d)	14.5 ±2.0	14.0 ±0.3	13.9 ±1.0

7.3.6 5-oxoproline Excretion

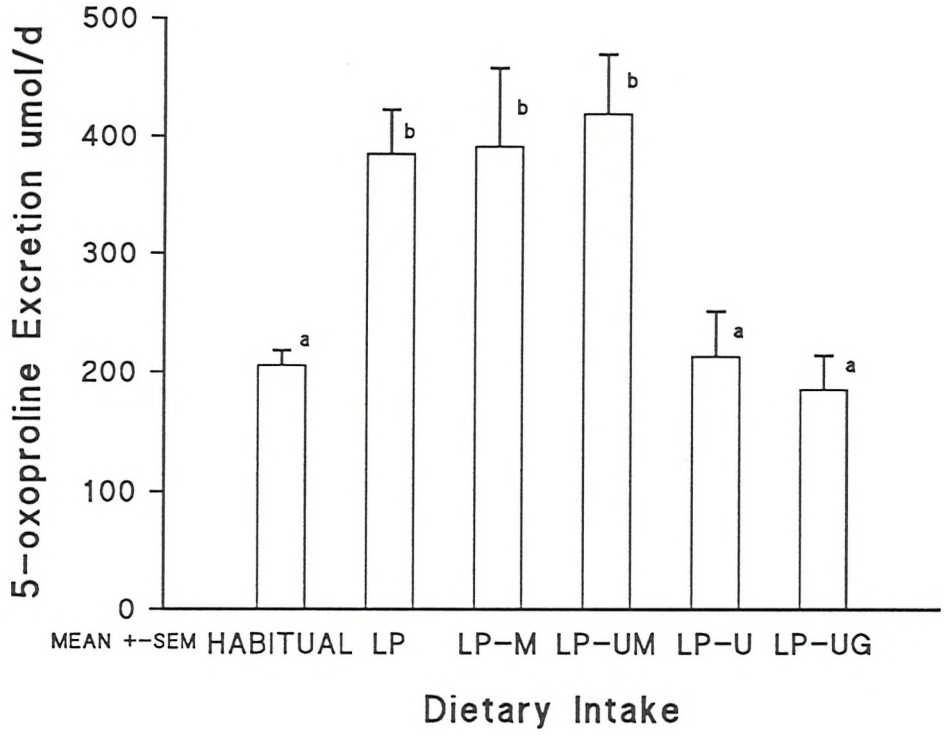
Urinary excretion was measured in samples taken from the male and female studies as a measure of the glycine insufficiency of the different diets and their supplements. Excretion on day 1 was taken to reflect the amount of 5-oxoproline excreted on a habitual diet. Figure 7.5 shows the 5-oxoproline excretion for the diets of the males studies compared to their habitual excretion and Figure 7.6 shows the same for the five female feeding protocols. Individual data is given in appendix 7.

Figure 7.5 Urinary 5-oxoproline excretion for six adult male subjects on four differing dietary regimens with varied nitrogen content compared to their habitual excretion.



With the male subjects, the REF diet containing 70g protein/day showed a similar level of 5-oxoproline excretion as the habitual level, 223.8 ±25 µmol/d compared to the habitual 243.4 ±30 µmol/d, indicating that this diet is sufficient in glycine. When intake was restricted to 30g protein/day, the excretion of 5-oxoproline rose to 510.7 ±97 µmol/d. The smaller 6.9g urea supplement partially reduced this deficit in glycine as excretion of 5-oxoproline fell to 392.9 ±34 µmol/d, although this was not significantly different to that on the LP diet. When the larger 13.7g urea supplement was given, 5-oxoproline excretion fell to 277.7 ±16 µmol/d, a value similar to that of the REF dietary intake. This suggests that the urea supplement was hydrolysed in the colon and the recycled nitrogen contributed to the body's glycine pool to reduce the dietary deficit.

Figure 7.6 Urinary 5-oxoproline excretion in six adult females consuming diets with varied nitrogen and sulphur amino acid contents, compared to their habitual excretion.



In the series of female feeding studies the results showed a similar pattern. The habitual urinary excretion level of 5-oxoproline was 205.4 ±13 µmol/d, a level similar to that seen in the males. When a LP diet of 26g protein/day was consumed, 5-oxoproline excretion rose to 385 ±38 µmol/d, which reflected that the diet was deficient in glycine. Addition of methionine, either alone or with urea, had no beneficial effect on this insufficiency with 5-oxoproline excretion staying elevated (being 391.0 ±67 µmol/d on the LP-M diet and 419 ±50 µmol/d on the LP-UM diet). When the urea was supplemented alone,

excretion fell to levels equivalent to habitual at 213.0 ±38 µmol/d, which backs up the evidence of increased colonic salvage in the women on this level of urea supplement. When this non-essential nitrogen supplement was partially as glycine (LP-UG), excretion of 5-oxoproline fell further to 185.8 ±28 µmol/d. This was not significantly different to the LP-U intake due to the large inter-individual variation and was a similar level of excretion to that seen habitually.

7.4 Discussion

In this series of feeding studies, the comparative effects of urea or glycine as non-essential nitrogen sources for colonic salvage were investigated, both improving nitrogen balance. The excretion of 5-oxoproline as a biological marker of glycine insufficiency gave a further insight into nitrogen metabolism in the face of protein restriction, demonstrating different mechanisms for improving nitrogen balance for the two nitrogen sources.

The experimental design which enabled us to examine the influence of the non-essential amino acid glycine on urea kinetics, was to include a further feeding protocol to the four studies reported in the last chapter. Hence, in this chapter, the LP and LP-U diets reported previously have been compared to one extra feeding study, that including a supplement of urea and glycine, LP-UG. The protocol used was the same format as in the previous two chapters and the same group of women undertook the studies as in chapter 6. The LP diet was a 26g protein/day intake, as described in Table 6.2 and the supplement was again given in a flavoured drink. The LP-UG supplement was calculated to include 3.2gN as given in the 6.9g urea supplement. Half of the supplement was as urea (3.4g) and the rest as glycine (8.6g $\equiv$ 1.6gN), in order that there was a source of urea available for the colonic bacteria.

It has already been demonstrated that non-essential nitrogen can improve nitrogen balance when added to a diet low in protein and that a source of non-essential nitrogen is essential for synthesis of non-essential amino acids when nitrogen from essential amino acids is limited. However, non-essential nitrogen sources vary in their effectiveness. Attempts to compare the effectiveness of sources of non-essential nitrogen with different amino acid patterns and different levels of total nitrogen content have led to a ranking of non-essential nitrogen sources (Swendseid *et al*

1960, Clarke *et al* 1963, Watts *et al* 1964, Kies *et al* 1965b, 1972b, 1973a, Jackson 1995). When lower amounts of non-essential nitrogen are fed the source becomes more significant (Kies & Fox 1972b).

In the above studies, it becomes clear that the most effective source of non-essential nitrogen are the non-essential amino acids as these are the desired product in the body. Other sources are less effective as they have to undergo metabolic exchange to satisfy the bodies demands for non-essential amino acids. Glycine, in combination with other non-essential nitrogen sources such as glutamate, diammonium citrate or urea, is consequently more effective than diammonium citrate or urea alone. However, some care should be taken with these conclusions as they are drawn from many studies with differing conditions and total nitrogen intakes.

Hence, as the previous chapter suggests that methionine is unlikely to be a limiting factor under our particular experimental conditions, we then considered if non-essential amino acids were limiting, especially glycine due to its central role in metabolism. Glycine is also important in the process of urea salvage. If the hydrolysed nitrogen is taken back into the body as ammonia, this passes to the liver where it is incorporated into the amino acid pool through amination of the non-essential amino acids ie. as glutamate or the glycine/serine exchange. Hence, glycine is important if ammonia is to be incorporated into synthetic pathways rather than simply returning to the urea cycle. The results demonstrated that supplemental urea at the level of 3.2gN/day was utilised by the women on the LP-U intake, and an apparent increase in urea salvage was able to improve nitrogen balance. Only a small proportion of recycled label is excreted as $^{15}N^{14}N$ urea so the possibility of glycine availability limiting the amination of non-essential amino acids is unlikely.

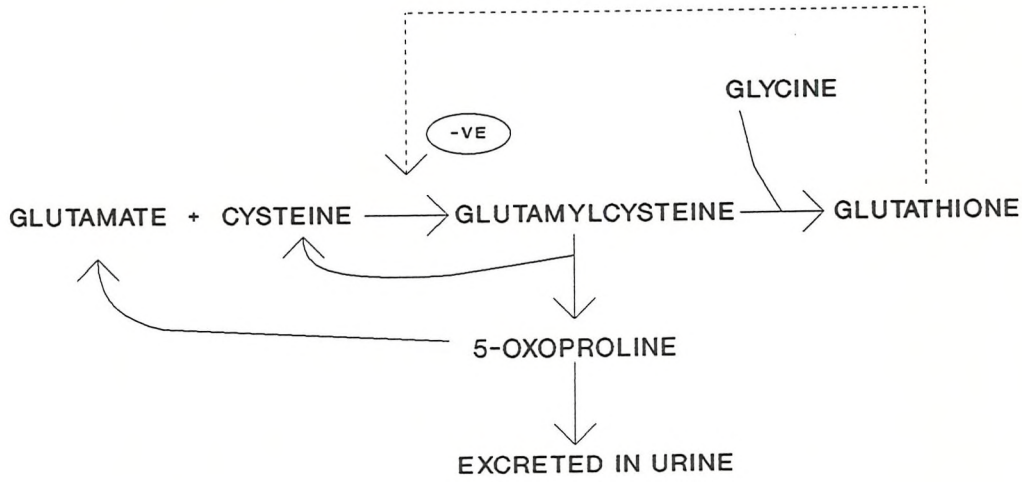
A similar improvement in nitrogen balance was observed with the LP-UG diet. However, there was no increase in urea hydrolysis which suggests that a different mechanism is operating. One theory is that no increase in hydrolysis is

necessary because the non-essential amino acids are being replace directly by the glycine supplement and no exchange of nitrogen is required. The LP-UG supplement did not improve nitrogen balance to equilibrium and there was no beneficial influence when compared to urea supplemented alone (LP-U). To look at these findings more closely, glycine status was examined using the excretion of 5-oxoproline.

5-oxoproline is an intermediate in the τ-glutamyl cycle which leads to the formation of glutathione (a simplified version is shown in Figure 7.7) and is the anhydride of glutamic acid. If there is insufficient glycine present for the formation of glutathione, there is a release on the feedback inhibition on the production of glutamylcysteine and this follows the alternate pathway to form 5-oxoproline. Jackson first suggested that under certain circumstances, urinary 5-oxoproline might provide a useful index of glycine insufficiency (Jackson *et al* 1987). He showed that following a large oral dose of sodium benzoate in adult humans, there was an increase in urinary 5-oxoproline over the next four hours as the glycine pool became depleted. The increase in urine was reduced by supplementary glycine.

The excretion of 5-oxoproline was measured on day 5 of all of the dietary protocols in both the male and female studies. The male data (Figure 7.5) demonstrates that the LP diet was clearly deficient in glycine as compared to habitual excretion of 5-oxoproline and that on the REF intake. This is due to a decrease in glycine synthesis on low protein intakes (Gersovitz *et al* 1980). With urea supplements, an increase in urea salvage decreased the excretion of 5-oxoproline, suggesting that the non-essential nitrogen was contributing to the bodies glycine pool via colonic salvage, either as glycine formation in the body from ammonia or incorporation of glycine produced by the colonic bacteria.

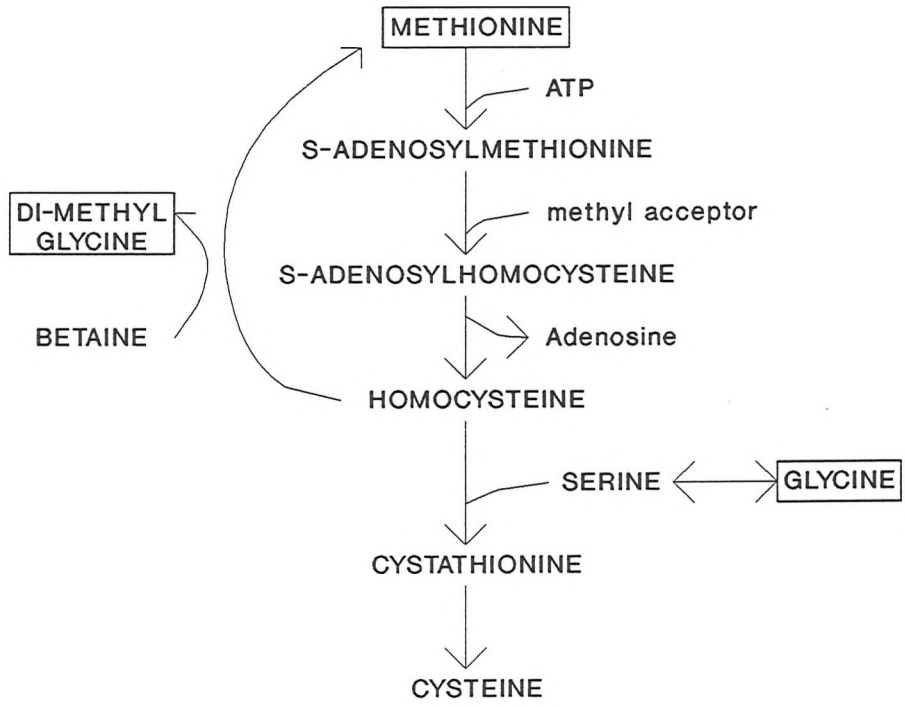
Figure 7.7 The formation of 5-oxoproline in the τ-glutamyl cycle.



The female data (Figure 7.6) demonstrates two findings. Firstly, the urea supplement (LP-U) increased urea hydrolysis to improve the deficit in glycine, as seen in the males. Also, the glycine and urea supplement in the LP-UG intake improved glycine status by directly supplementing glycine.

The results also demonstrate the link between glycine metabolism and that of the sulphur amino acids (Figure 7.8) and gives more information as to the influence of the methionine supplements described in chapter 6. The supplementation of methionine, both alone and with urea, did not improve glycine status. Conversely, if glycine was deficient, then methionine may build up as it cannot pass down the transsulphuration pathway due to a lack of glycine-serine exchange as glycine is channelled to other products.

Figure 7.8 The interaction of methionine and glycine metabolism in the transsulphuration pathway.



This leads to methionine toxicity which results in an increase in amino acid excretion and hence, a negative nitrogen balance (Klain *et al* 1963). This toxicity is alleviated by supplemental glycine (Roth & Allison 1949, Klain *et al* 1963, Benevenga & Harper 1967), presumably as methionine is then converted to cysteine. Hence, a further experiment would have been interesting to investigate whether addition of glycine to the LP-UM diet would have overcome the negative influence of the methionine and returned nitrogen balance to that found with the LP-U diet.

In conclusion, it appears that supplemental glycine can improve nitrogen balance and reduce a deficit in glycine perhaps by direct replacement rather than recycling the nitrogen via urea salvage. A supplement of urea and glycine was no more efficient than one of isonitrogenous urea during the conditions used in our experiments. These findings appear to be in contrast to studies described in the literature and whether this was due to the nitrogen level of the supplement or the level of nitrogen intake in the low protein diet is unclear. The 5-oxoproline data shows that in circumstances of restricted protein intake, the non-essential amino acids, particularly glycine are limiting and replacement of these allows the individual to improve nitrogen balance.

7.5 Summary

The influence of glycine of the urea salvage system was investigated by comparing the supplementation of two sources on non-essential nitrogen to a low protein diet in a group of young adult women. A supplemental source of urea was utilised to improve nitrogen balance by increasing urea hydrolysis and reincorporating the nitrogen into the body pool. With supplemental glycine, nitrogen balance was improved by replacing the deficit in glycine directly without an increase in urea salvage. This demonstrates the essentiality of glycine and a non-essential nitrogen source to the body under conditions when either the demand is increased or supply is restricted.

CHAPTER 8

GENERAL DISCUSSION

The principle aim of this thesis was to gain an understanding of the controlling factors which allow the body to adapt to low protein intakes by nitrogen recycling in the colon. The use of stable isotope labelling enabled the measurement of urea kinetics in free living individuals consuming diets with a protein content below the minimum requirement. From this basic experimental model, the effects of supplemental nitrogen sources could be examined.

It was found that the primary influence on urea salvage was total nitrogen intake rather than simply the protein content of the diet. The colonic salvage system responded to the daily cycles of nitrogen intake. During the overnight fast, urea hydrolysis increased thereby compensating in part for a restricted dietary intake.

Addition of dietary urea supplements to an inadequate protein intake improved nitrogen balance by increasing the rate of urea hydrolysis in the colon and reincorporating the nitrogen back into the body. This appeared to be stimulated by an increased urea appearance in the body urea pool. However, substantial amounts of this non-essential nitrogen source were required to boost the start of the urea salvage system and hence, the possibility of other limiting nutrients was examined.

Based upon the amino acid pattern of the diet, it was considered that the sulphur amino acids might be a limiting factor, being the most limiting amino acids in the experimental diet. However, the improvements to nitrogen balance shown by Kies in the 1960's was not replicated and methionine supplementation did not improve the nitrogen deficit and in fact removed any beneficial effect of the supplemental urea.

A further source of non-essential nitrogen, in the form of glycine, was examined as this had been shown to be a more efficient source of nitrogen for improving nitrogen balance. It was found that glycine improved nitrogen balance at a

similar magnitude to that of urea, demonstrating the essentiality of a dietary source of glycine under these conditions. These conclusions were further reinforced by the resulting urinary excretion of 5-oxoproline. It appeared, however, that the improvement in nitrogen balance was achieved by direct supplementation rather than indirectly via enhanced urea salvage.

Before any conclusions can be drawn from the results presented in this thesis, the validity of the data should be discussed. The results presented in chapter 4 demonstrated the importance of 24 hour measurements of urea kinetics in order to take into account diurnal changes in urea excretion and hydrolysis. The influence on these parameters by expansion and contraction of the urea pool could also be dispelled if measurement are taken over a 24 hour period.

Inter-individual variability was considerable amongst the male and female subjects, demonstrating that different individuals could respond to varying extent to the changing nitrogen supplements (Table 8.1). However, these variations were not beyond those often experienced in studies using humans subjects (Hibbert & Jackson 1991, McClelland 1994). The rates of urea hydrolysis showed the greatest variation between subjects as individuals responded to different degrees to the non-essential nitrogen supplements. The reasons why individuals have differing rates of urea salvage under similar conditions are unknown. All the subjects were free living in their own homes and limited checks were maintained over their compliance to the study protocol. All the subjects were highly motivated and undertook a series of studies. Although, it has been demonstrated that the order in which studies are carried out may influence the results obtained (Atinmo *et al* 1988), there was no evidence to suggest that variability decreased as more studies were completed by the individual. Three weeks were left between studies to allow individuals to return to their habitual nitrogen status rather than only three days in the studies of Atinmo.

Table 8.1 Inter-individual variation in measures of urea kinetics in young men and women consuming low protein diets with varied nitrogen supplements (expressed as coefficient of variation %).

PROTOCOL	INTAKE	Au	Pu	T	Eu
MALES					
REF	8.0	31.8	31.8	62.9	17.8
LP	8.0	15.4	15.4	51.2	15.9
LP-U1	8.0	19.4	26.9	113.6	20.1
LP-U2	8.0	15.4	26.2	38.3	20.0
FEMALES					
LP	13.7	22.8	22.8	54.2	19.9
LP-M	13.7	10.7	10.7	98.2	35.3
LP-U	13.7	15.0	23.1	89.4	27.1
LP-UM	13.7	26.1	39.5	112.6	21.1
LP-UG	13.7	22.0	25.3	73.9	21.2

Another way to examine our confidence in the present results is to compare them directly with the findings of previous reported studies. Hence in Table 8.2, urea kinetics from studies using males are compared and it can be seen that for intakes of similar magnitude, the rates of urea production are remarkably consistent. The rates of urea excretion and hydrolysis found in the present study fit in with the pattern seen in Figure 2.5 (Langran *et al* 1992, Danielsen & Jackson 1992) over a range of protein intakes. A similar agreement is seen with the studies carried out in females for this thesis and other urea kinetic measurements in females (Table 8.3).

Table 8.2 Summary of values of urea kinetics obtained in studies carried out in normal adult men on varying protein intakes (values expressed as mgN/kg/d).

	INTAKE	Au	Pu	T	Eu
DANIELSEN	165	199	199	80	118
LANGRAN	149	194	194	92	101
PRESENT	147	209	209	83	126
LANGRAN	76	172	172	115	57
DANIELSEN	68	123	123	58	64
PRESENT	62	118	118	34	84
PRESENT	62(84)=146	204	121	79	125
PRESENT	62(42)=104	166	124	35	131

( ) nitrogen from dietary urea supplements

Table 8.3 Summary of the values for urea kinetics obtained in studies carried out in normal adult women on varying protein intakes (values expressed as mgN/kg/d).

	INTAKE	Au	Pu	T	Eu
McCLELLAND	181	200	200	76	124
FORRESTER	167	150	150	40	110
HIBBERT	231	198	198	55	143
McCLELLAND	153	186	186	74	111
BUNDY	156	182	182	71	111
PRESENT					
LP	69	88	88	20	68
LP-M	69(1)=70	84	84	18	69
LP-U	69(52)=121	144	91	32	113
LP-UM	69(53)=122	135	82	23	115
LP-UG	69(52)=121	128	101	21	107

( ) nitrogen from dietary supplements

When all these studies are considered as a whole then a picture begins to emerge of the metabolic control on the urea salvage system and in particular, the switch point which enables successful adaptation. Availability of urea is an important determinant for the rate of urea salvage. A primary control is the appearance of urea in the body pool (normally from endogenous production) which, in turn is dependent on the nitrogen content of the diet. The results from these studies and those of Kies suggest that nitrogen balance is achieved only when the total nitrogen intake is above a certain threshold dependent on essential amino acid intake (Kies *et al* 1963, 1965b, 1965c, 1972b). This threshold lies between 8.0-11.2gN/day. There is an underlying determinant for urea salvage in an individual of the level at which urea production and hydrolysis operate. Below a certain level of urea production, hydrolysis cannot be maintained at rates which sustain nitrogen balance. It appears that the critical level for adaptation by urea salvage is when urea appearance in the pool is about 160 mgN/kg/d. When the rate of urea appearance approaches 200mgN/kg/d, either through intake or urea supplementation, the individual is in nitrogen balance. The rate of urea salvage could also be influenced by the factors which set the level of urea appearance. Appearance is maintained above the critical point by the sum of nitrogen intake and urea hydrolysis. From the results in Table 8.2, it is clear that nitrogen equilibrium was only achieved when intake plus hydrolysis exceeded 150 mgN/kg/d. This critical rate of urea appearance is set at a similar level in the female subjects when expressed per unit body weight, with urea hydrolysis leading to an improved nitrogen balance at levels of urea appearance above 150 mgN/kg/d. The most effective supplement in the females is that seen with the LP-u intake with values for urea appearance at 144 mgN/kg/d, ie. approaching the critical point.

However, urea salvage does not function in isolation. It is part of a dynamic metabolism and hence, any one of the factors which control the rate of salvage (Figure 2.4) could have an

influence. For example, other nutrients rather than nitrogen may be influential, particularly the energy content of the experimental diet.

Energy intake has a very strong interaction with nitrogen metabolism. Nitrogen balance has long been shown to be influenced by energy balance (Munro 1951, Calloway & Spector 1954) and that at a given level of protein intake, addition of energy to the diet improves nitrogen balance (Garza *et al* 1978, Iyengar *et al* 1979). Evidence points to the oxidation of amino acids for energy, which accounts for about 15% of daily energy requirements, as a major drive for urea production. (Jackson 1994a). Therefore, it is not surprising that the protein:energy ratio influences urea kinetics (Jackson 1990b). The experiments shown in this thesis provided energy at a level of approx 130 kJ/kg/d which was sufficient to supply the requirement level of energy for a sedentary lifestyle, ie. BMR (measured by indirect calorimetry) x1.4 (DRV, HMSO 1991). However, if this was not sufficient for some of the more active subjects, who were free living during the studies and whose exercise was not regulated, then the salvaging of nitrogen in the colon may have been influence by the availability of energy. This is an area which needs further research.

Other controls on the salvage of urea may also have been influenced by the experimental conditions. In particular, addition of urea and the expansion of the body urea pool may have influenced the closely regulated systems for maintaining acid-base balance and body fluid homeostasis.

Another factor which may account for differences in urea salvage, both between individuals on comparable intakes and between the male and female subjects, is body composition. In the past, it has been concluded that body composition can influence protein metabolism and that obese people can survive a fast for longer by conserving body nitrogen by excreting less in the urine (Henry 1991). A recent study found that this alteration in nitrogen excretion in the obese was reflected in urea salvage in the colon as on a similar intake to normal

women, obese women had a higher rate of urea production and hydrolysis (Child 1995). So a number of correlations were carried out on the data gathered in this thesis from the male and female subjects to examine the relationship between urea kinetic parameters and measures of body composition (Figure 8.1). A clear relationship can be seen in these two parameters of urea kinetics with an increase in body weight even using two groups of lean individuals and with the various dietary manipulations. The closest relationship is seen with resting energy expenditure, again demonstrating the important link between nitrogen and energy metabolism (Figure 8.2). It also suggests that the metabolism of the colonic microflora may be linked to the basal metabolic rate of the host individual.

Figure 8.1 The relationships between urea production and hydrolysis and measures of body composition in young adults.

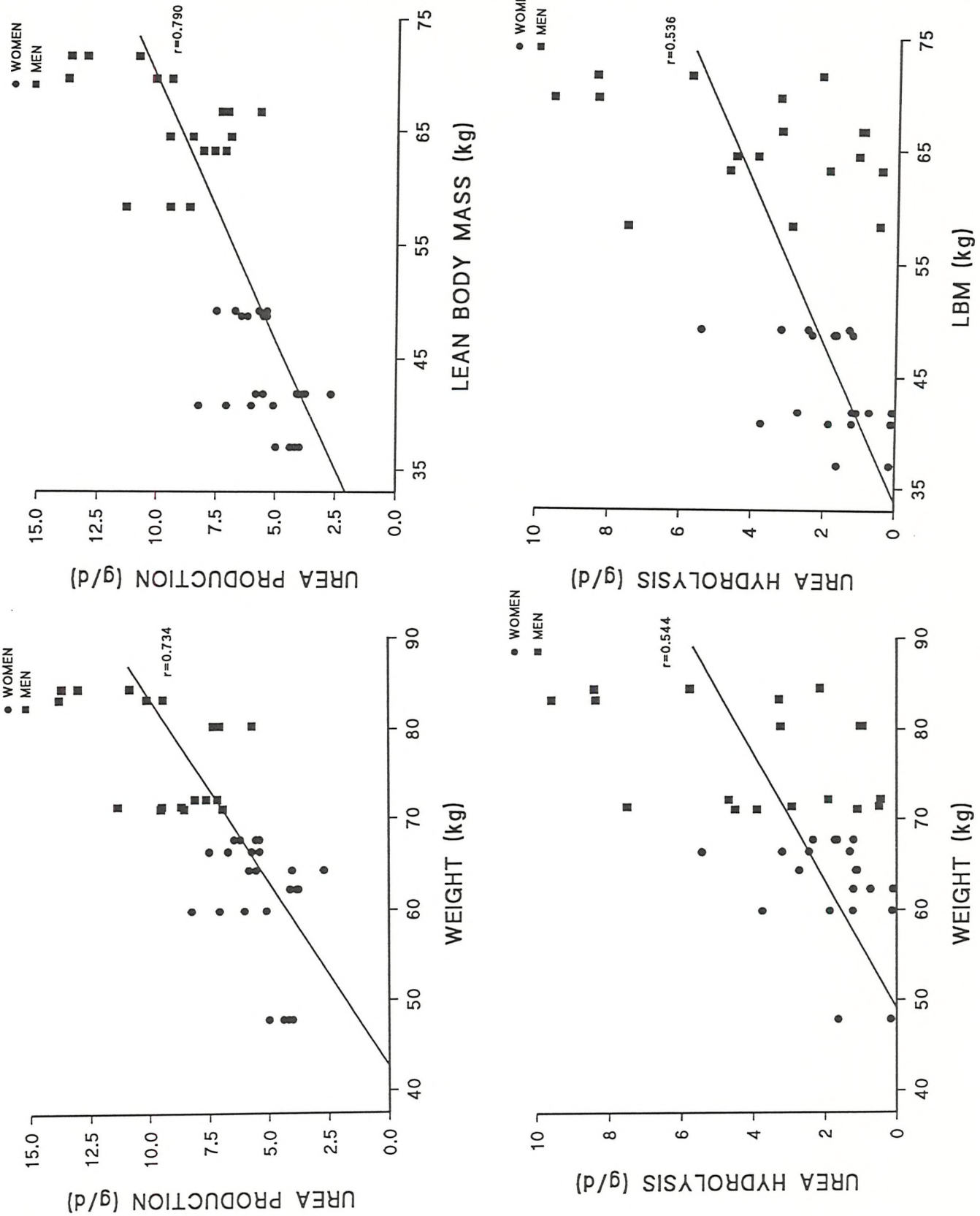
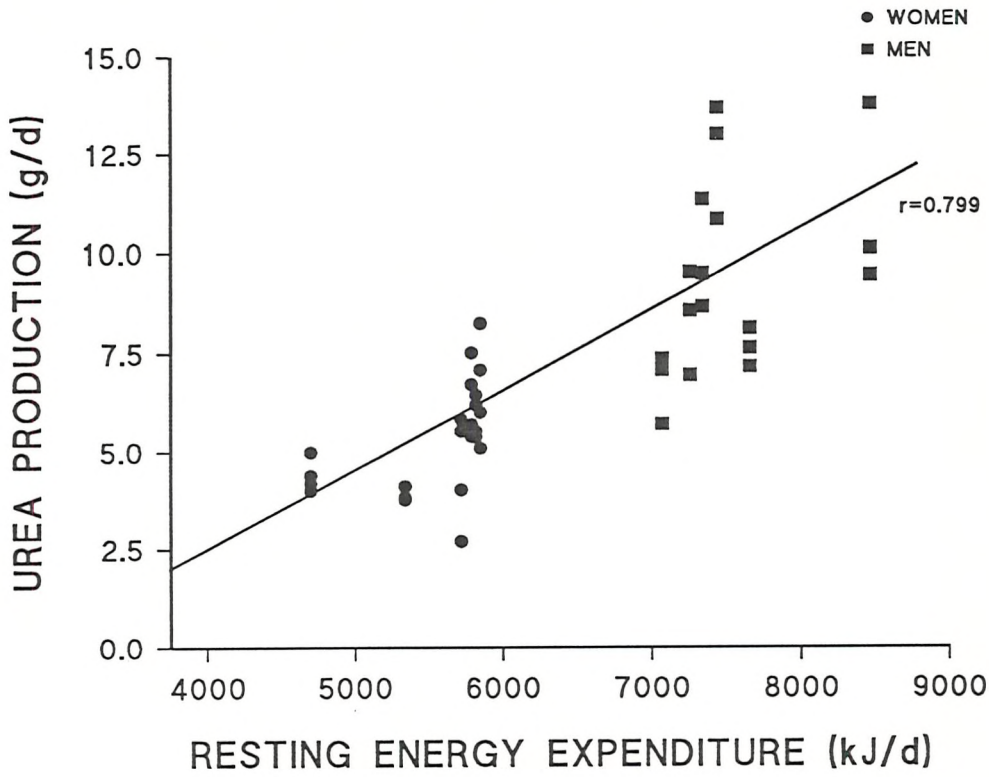
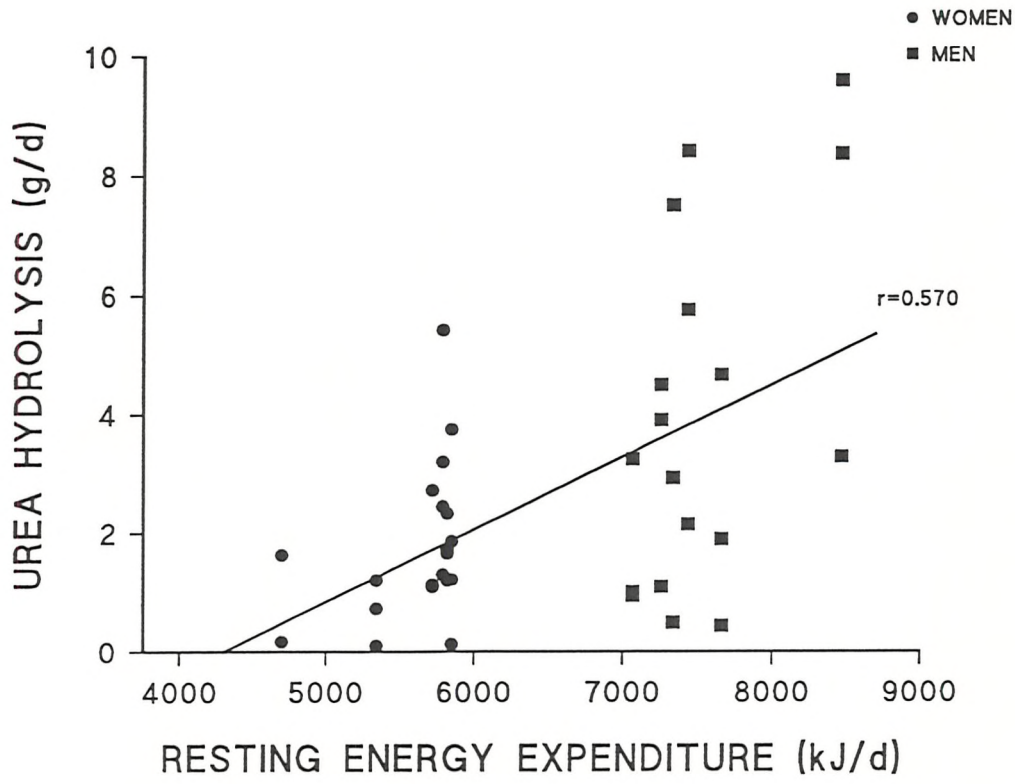


Figure 8.2 The relationship between urea production and hydrolysis and resting energy expenditure in young adults.





By following the ideas of the classical experiments of Constance Kies, the importance of non-essential nitrogen could be examined using a more detailed technique than body balance studies. Kies gave a clear picture of the relationships between the amount of protein in the diet and the ratio of essential amino acids to total nitrogen required to meet nitrogen balance in normal adults. From the data generated, conclusions can be drawn about the relative efficiency of various nitrogen sources, on the assumption that there is a metabolic demand for a defined pattern (Jackson 1995). The closer the amino acids in the diet are to this ideal pattern, the less amino acids are needed to achieve balance. Nitrogen equilibrium can be achieved at 3-4g nitrogen/day if the diets provides two-three times Roses minimal requirements (Rose 1957) with a low level of non-essential amino acids. If large amounts of non-essential nitrogen are provided in the diet, balance can be achieved at 6-8gN with Roses minimal requirements, as was the case in many of Kies experiments (Kies *et al* 1963, 1965b, 1965c, 1972). Further, non-essential nitrogen can substitute for non-essential amino acids but the total nitrogen intake may have to increase to 12 gN/day. This seems to be the case with studies in this thesis, with balance only being achieved when nitrogen intake was 11.2 gN/day. Different sources of non-essential nitrogen substitute with greater efficiency for non-essential amino acids (see rank order in Table 2.2). The results from the female subjects in chapter 7 tend to agree with this ranking with respect to glycine/urea and urea, both being relatively inefficient but able to improve nitrogen balance. The technique of urea kinetics using the stable isotope $^{15}N^{15}N$ urea enabled the mechanisms behind these balance studies to be measured dynamically.

To put the conclusions from these studies in context, they underline two integral points in nitrogen metabolism. The first is the importance of non-essential nitrogen in meeting an individuals requirements for nitrogen. Most recommendations for protein requirements focus on the pattern of essential amino

acids necessary in the diet. Although the essential amino acids are of primary importance, the body's requirement for them can be altered by the content of non-essential nitrogen. Addition of non-essential nitrogen to a low protein diet can improve the nitrogen balance of an individual. It may be more appropriate to consider this nitrogen source as 'non-specific' as the term 'non-essential' undermines its importance in human nutrition.

Integral to the role of non-specific nitrogen in metabolism is the urea salvage system in the colon. Nitrogen is recycled back into the body pool via the action of the colonic microflora. The form of the nitrogen returning to the body is only now beginning to be examined and there is some evidence that bacterially formed amino acids are absorbed into the body. The urea-nitrogen salvage is a part of the body's adaptive mechanism to a lowered dietary intake, with an increase in urea hydrolysis and subsequent nitrogen recycling compensating for a reduction in intake. The system has a lower limit at 35g protein/day in men which can be taken to be the absolute minimal requirement level. The factors which set and control this 'switch point' in individuals are complex. However, this thesis goes some way to examine the influence of nitrogen intake on this control mechanism. The nitrogen intake from the diet determines the appearance of urea in the body pool which, in turn, regulates the rate of urea hydrolysis in the colon.

Urea salvage is an integral part of nitrogen metabolism in all metabolic conditions; being of principle importance during protein restriction. If the metabolism of amino acids and nitrogen is to be fully understood in order to give informed recommendations for protein intakes for populations, then urea salvage can no longer be considered a 'futile cycle' and should be included in all models for amino acid kinetics.

APPENDIX 1 Nutritional Analysis of the Experimental Diets.

Nutritional content was assessed using Comp-eat computer analysis.

1A. The REFerence (70g protein/day) Diet (Chapters 4 & 5).

NUTRIENT	Amount	RDA	% RDA
Energy kcal	2662	980	272
Energy kJ	11154		
Protein g	70.14	24.50	286
Fat g	132.37		
Polyunsats g	15.14		
Monounsats g	35.49		
Saturates g	43.91		
Carbohydrate	319.14		
Sugar g	159.19		
Starch g	150.89		
Fibre -NSP g	13.85		
Calcium mg	900	600	150
Phosphorus	1370	360	381
Magnesium mg	317	70	452
Sodium mg	3650	750	487
Potassium mg	3250	1250	255
Chloride mg	4527		
Iron mg	29.29	6.0	488
Zinc mg	8.71	10	87
Copper mg	1.61	0.7	230
Selenium µg	5.38		
Iodine µg	98.76		
Thiamin mg	1.64	0.3	545
Riboflavin	2.12	0.4	531
Nic. Acid mg	35.49	5.0	710
Vit. B6 mg	2.36	0.6	393
Vit. B12 µg	3.89	1.5	259
Folate µg	298	50	595
Vit. C mg	105	20	527
Vit. A µg	1010	450	224
Vit. D µg	4.18	7.5	56
Vit. E mg	125	4.0	182
Cholesterol	931.48		

NSP = non-starch polysaccharide
Vit. = vitamin

1B. The Low Protein (30g protein/day) Diet used in the male studies. (Chapter 5)

NUTRIENT	Amount	RDA	% RDA
Energy kcal	2718	980	277
Energy kJ	11345		
Protein g	24.50	24.50	**100**
Fat g	172.60		
Polyunsats g	14.14		
Monounsats g	43.97		
Saturates g	69.97		
Carbohydrate	284.72		
Sugar g	187.75		
Starch g	76.93		
Fibre -NSP g	6.84		
Calcium mg	492	600	**82**
Phosphorus	508	360	141
Magnesium mg	213	70	305
Sodium mg	2503	750	334
Potassium mg	2825	1275	222
Chloride mg	2513		
Iron mg	9.45	6.0	157
Zinc mg	3.93	10	**39**
Copper mg	1.09	0.7	155
Selenium µg	17.90		
Iodine µg	41.44		
Thiamin mg	1.13	0.3	375
Riboflavin	1.24	0.4	309
Nic. Acid mg	16.33	5.0	327
Vit. B6 mg	1.54	0.6	257
Vit. B12 µg	1.28	1.5	**85**
Folate µg	271	50	543
Vit. C mg	173	20	863
Vit. A µg	3167	450	704
Vit. D µg	4.78	7.5	**64**
Vit. E mg	8.50	4.0	213
Cholesterol	255		

1C. The Low Protein (26g protein/day) Diet used in the female studies (Chapters 6 & 7).

NUTRIENT	Amount	RNI	% RNI
Energy kcal	2010		
Energy kJ	8433		
Protein g	23.99	45.00	**53.31**
Fat g	108.93	73.69	147.82
Polyunsats g	15.23	13.40	113.69
Monounsats g	23.25		
Saturates g	19.71	22.33	**88.26**
Carbohydrate	249.64	252.27	**98.96**
Sugar g	137.98		
Starch g	81.56		
Fibre -NSP g	7.05	24.00	**29.40**
Calcium mg	480	700	**68.56**
Phosphorus	420	550	**76.35**
Magnesium mg	175	270	**64.73**
Sodium mg	3075	1600	191.07
Potassium mg	2925	3500	**83.56**
Chloride mg	2637	2500	105.47
Iron mg	9.41	14.80	**63.61**
Zinc mg	3.45	7.0	**49.31**
Copper mg	0.88	1.0	**88.41**
Selenium µg	22.80	60	**38.00**
Iodine µg	26.30	140	**18.79**
Thiamin mg	1.07	0.8	133.38
Riboflavin	1.05	1.10	**95.49**
Nic. Acid mg	17.17	13.26	129.42
Vit. B6 mg	1.08	0.36	301.03
Vit. B12 µg	1.20	1.50	**80.00**
Folate µg	227	200	113.57
Vit. C mg	110	40	274.85
Vit. A µg	3219	600	536.47
Vit. D µg	2.82		
Vit. E mg	5.71	6.09	93.71
Cholesterol	39		

* updated version of Comp-eat

1D. Amino Acid Analysis of the Experimental Diets.

mg	REF diet 70g P	LP diet 30g P	LP diet 26g P	RNI FAO 1985 men/women
Isoleucine	1884	808	753	715/585
Leucine	3279	1365	1340	1045/855
Lysine	2633	941	818	880/720
Methionine	952	**372**	**358**	
Cysteine	641	**305**	**280**	935/765
Phenylalanine	1937	894	837	
Tyrosine	1474	609	571	1045/855
Threonine	1646	657	639	495/405
Tryptophan	704	**256**	238	275/225
Valine	2301	1015	964	715/585
Arginine	2021	905	779	
Histidine	1202	475	408	880/720
Alanine	1798	790	670	
Aspartic Ac.	3563	1850	1557	
Glutamic Ac.	9151	4035	4011	
Glycine	1574	759	554	
Proline	3437	1547	1492	
Serine	2078	925	880	

Ac. = acid
bold no. = amino acid below recommended levels (FAO 1985).

APPENDIX 2 **Individual Data for Urea Kinetics in Seven Adult Males measured over 27 hours to demonstrate diurnal variation.**

2A. Urea production in seven adult males fed 70g protein/day measured in three hour intervals over 27 hours (mgN/kg/h).

Time / Subject	06.00 - 09.00	09.00 - 12.00	12.00 - 15.00	15.00 - 18.00	18.00 - 21.00
1	5.892	6.056	7.736	7.051	6.666
2	8.176	11.105	7.065	6.923	6.235
3	4.563	4.970	4.732	5.873	5.351
4	11.309	9.159	14.313	8.349	10.603
5	6.159	7.590	8.069	8.271	8.891
6	6.509	4.899	6.046	7.387	7.647
7	10.411	7.793	11.292	7.616	7.137
mean	7.574	7.367	7.308	7.353	7.504
±SEM	0.944	0.859	0.463	0.323	0.666

Time / Subject	21.00 - 00.00	00.00 - 03.00	03.00 - 06.00	06.00 - 09.00
1	8.441	6.691	8.625	9.055
2	7.075	7.434	6.682	5.820
3	7.346	4.778	7.269	3.963
4	10.110	6.936	6.937	5.655
5	11.878	12.019	9.479	13.160
6	7.039	4.516	5.621	5.621
7	7.526	12.683	5.848	5.830
mean	8.448	7.814	7.209	6.986
±SEM	0.698	1.255	0.532	1.180

2B. Urea Excretion in seven adult males fed 70g protein/day measured in three hourly intervals over 27 hours (mgN/kg/h).

Time / Subject	06.00 - 09.00	09.00 - 12.00	12.00 - 15.00	15.00 - 18.00	18.00 - 21.00
1	5.291	3.975	5.817	5.360	4.917
2	5.105	0.460	1.478	15.272	2.943
3	2.606	2.715	3.015	7.176	7.776
4	4.610	5.898	4.799	5.745	8.375
5	2.535	5.042	7.605	4.370	4.384
6	2.910	2.873	3.172	3.719	6.405
7	2.877	3.349	3.365	5.063	6.415
mean	3.705	3.473	4.179	6.672	5.939
±SEM	0.468	0.666	0.774	0.1491	0.757

Time / Subject	21.00 - 00.00	00.00 - 03.00	03.00 - 06.00	06.00 - 09.00
1	5.416	4.321	4.584	1.565
2	3.403	5.202	5.244	4.909
3	5.525	2.292	1.187	1.978
4	8.002	5.780	3.121	5.000
5	9.244	4.566	4.510	4.650
6	1.169	6.517	3.731	3.147
7	7.248	3.726	2.752	3.616
mean	5.715	4.629	3.590	3.552
±SEM	1.048	0.525	0.518	0.529

APPENDIX 3 **Individual Data for Urea Kinetics and Clearance in Six Adult Males consuming Diets with Different Nitrogen Content to Demonstrate the Influence of Non-Essential Nitrogen on Urea Salvage.**

3A Urinary Nitrogen Excretion in six adult males consuming diets of different nitrogen content (mgN/kg/d).

DIET EXCRETION mgN/kg/d	REF 11.2gN	LP 4.8gN	LP-U1 8.0gN	LP-U2 11.2gN
DAY 1	-	130.312	222.235	189.158
	78.244	73.135	-	-
	182.314	100.675	105.295	128.777
	186.809	156.487	180.632	216.802
	143.277	143.744	141.728	190.727
	106.915	170.016	191.354	141.331
MEAN	139.512	129.062	168.249	173.359
±SEM	21.094	14.810	20.333	16.510
DAY 4	133.400	171.938	191.132	217.678
	159.014	88.100	135.598	155.766
	122.432	93.335	144.562	73.126
	171.491	105.910	134.979	149.764
	162.924	154.356	152.128	114.241
	182.487	105.811	172.445	207.942
MEAN	155.291	119.908	155.141	169.078
±SEM	9.369	14.150	9.136	19.274
DAY 5	92.159	92.519	150.162	187.826
	102.866	168.975	148.701	191.841
	125.653	82.047	121.146	149.250
	140.833	108.464	196.939	175.182
	131.571	81.197	162.156	169.637
	145.472	113.839	89.624	119.798
MEAN	123.092	107.840	144.788	165.589
±SEM	8.679	13.396	14.908	11.039
AVERAGE	114.155	134.875	171.274	203.744
DAY4/5	132.956	126.021	141.918	172.847
	123.933	88.068	133.634	108.650
	157.175	107.102	163.892	161.623
	148.291	120.212	156.809	140.093
	165.199	109.558	133.799	166.813
MEAN	140.292	114.306	150.221	158.962
±SEM	8.110	6.728	6.551	13.10

3B Urinary Urea Excretion in six adult males consuming diets of different nitrogen content (mgN/kg/d).

DIET EXCRETION mgN/kg/d	REF 11.2gN	LP 4.8gN	LP-U1 8.0gN	LP-U2 11.2gN
DAY 1	50.970	109.612	307.687	142.756
	98.720	60.942	-	113.699
	192.566	115.500	158.664	160.072
	130.745	157.045	170.803	205.130
	99.580	155.574	266.513	-
	92.102	132.251	122.861	-
MEAN	110.781	121.837	205.306	155.407
±SEM	19.395	14.586	34.922	19.140
DAY 2	61.316	80.997	232.216	125.512
	162.841	99.564	185.035	137.032
	158.777	61.679	140.959	91.691
	86.501	127.482	128.121	201.111
	99.020	154.832	166.359	121.261
	140.199	73.284	90.933	183.843
MEAN	118.109	99.640	157.271	143.408
±SEM	17.060	14.497	19.989	16.823
DAY 3	113.532	61.579	170.942	167.299
	168.734	73.812	168.861	141.069
	93.933	82.542	145.624	85.887
	91.005	93.818	97.340	125.757
	135.434	82.885	221.106	154.958
	152.674	77.177	99.876	123.209
MEAN	125.885	78.636	150.625	133.030
±SEM	12.955	4.398	19.281	11.679
DAY 4	166.787	79.515	137.008	114.114
	134.993	63.038	150.042	103.615
	160.695	83.189	91.247	55.300
	124.374	121.111	129.054	126.903
	80.392	112.143	169.916	116.625
	139.403	84.453	107.090	151.692
MEAN	126.107	90.575	130.726	111.375
±SEM	11.003	8.889	11.645	13.046
DAY 5	141.053	65.083	165.873	146.884
	90.380	79.015	149.887	162.968
	169.724	80.012	116.571	96.619
	133.239	81.548	116.726	150.165
	119.230	83.291	147.031	173.305
	93.731	74.229	87.786	99.279
MEAN	124.560	77.196	130.646	138.262
±SEM	12.294	2.726	11.703	13.266

DIET EXCRETION mgN/kg/d	REF 11.2gN	LP 4.8gN	LP-U1 8.0gN	LP-U2 11.2gN
AVERAGE	128.111	72.780	150.478	129.407
DAY4/5	114.174	70.494	149.970	131.313
	164.909	81.706	103.065	74.582
	128.511	102.648	123.301	137.759
	98.516	98.679	159.236	143.076
	118.089	79.682	98.081	127.233
MEAN	125.385	84.332	130.689	123.895
±SEM	9.093	5.461	10.738	10.146

3C Urinary Ammonia Excretion in six adult males consuming diets with different nitrogen content (mgN/kg/d).

DIET EXCRETION mgN/kg/d	REF 11.2gN	LP 4.8gN	LP-U1 8.0gN	LP-U2 11.2gN
DAY 1	3.920	6.801	12.493	8.019
	3.882	3.150	3.038	6.765
	4.496	5.072	3.933	6.271
	9.113	4.421	5.118	7.163
	2.857	6.779	4.230	8.526
	6.816	6.990	6.978	-
MEAN	5.181	5.536	5.965	7.409
±SEM	0.954	0.643	1.415	0.456
DAY 2	4.197	5.208	6.787	3.947
	6.850	5.654	9.226	7.286
	3.429	2.722	4.820	3.837
	3.605	4.113	4.740	6.513
	4.608	5.868	3.291	3.922
	6.642	4.988	3.371	3.893
MEAN	4.889	4.759	5.373	4.900
±SEM	0.613	0.478	0.929	0.640
DAY 3	5.983	3.864	3.643	5.249
	5.809	5.021	4.346	5.007
	4.616	1.942	3.753	4.257
	3.002	3.097	3.865	4.338
	4.664	3.515	3.655	3.641
	5.187	4.216	3.209	4.453
MEAN	4.877	3.609	3.745	4.491
±SEM	0.440	0.427	0.151	0.234
DAY 4	4.709	4.861	3.116	4.114
	6.090	4.487	7.820	4.852
	7.290	3.106	3.022	3.849
	3.050	3.298	3.712	3.652
	3.431	3.838	3.515	3.011
	6.953	4.291	3.905	4.677
MEAN	5.254	3.980	4.182	4.026
±SEM	0.735	0.282	0.741	0.278
DAY 5	6.177	4.307	4.058	3.573
	4.655	4.388	5.921	6.526
	8.753	4.472	4.317	4.580
	4.066	3.191	4.917	3.061
	5.476	2.773	5.448	2.815
	5.448	5.037	3.856	4.751
MEAN	5.763	4.028	4.753	4.218
±SEM	0.668	0.351	0.334	0.562

DIET EXCRETION mgN/kg/d	REF 11.2gN	LP 4.8gN	LP-U1 8.0gN	LP-U2 11.2gN
AVERAGE	5.394	4.602	3.556	3.862
DAY4/5	5.420	4.441	6.934	5.633
	7.973	3.743	3.626	4.190
	3.524	3.248	4.274	3.376
	4.385	3.341	4.417	2.920
	6.251	4.639	3.882	4.712
MEAN	5.491	4.002	4.448	4.116
±SEM	0.628	0.260	0.516	0.396

3D Apparent Nitrogen Balance in six adult males consuming diets with different nitrogen content (mgN/kg/d).

DIET	REF 11.2gN	LP 4.8gN	LP-U1 8.0gN	LP-U2 11.2gN
INTAKE	155.125	66.482	110.803	155.125
	157.525	66.496	111.576	157.525
	134.293	57.554	95.923	134.293
	132.388	56.738	94.563	132.388
	156.863	67.227	112.045	156.863
	139.303	59.701	99.502	139.303
LOSSES	138.155	158.875	195.274	227.744
-total	156.996	150.021	165.918	196.847
urinary	147.933	112.068	157.634	132.650
N + 24	181.175	131.102	187.892	185.623
mgN/kg/d	172.291	144.212	180.809	164.093
faecal &	189.199	133.558	157.799	190.813
misc. losses				
BALANCE	16.970	-92.393	-84.471	-72.619
	0.529	-83.075	-54.342	-39.322
	-13.640	-54.514	-61.711	1.643
	-48.787	-74.364	-93.329	-53.235
	-15.428	-76.985	-68.764	-7.230
	-49.896	-73.857	-58.297	-51.510
MEAN	-18.375	-75.865	-70.152	-37.046
±SEM	10.888	5.127	6.340	11.730

3E Urea Appearance and Production in six adult males consuming diets with different nitrogen content (mgN/kg/d).

DIET	REF 11.2gN	LP 4.8gN	LP-U1 8.0gN	LP-U2 11.2gN
APPEARANCE Au	171.487	99.237	156.644	194.321
	183.500	133.965	165.437	187.580
	332.981	121.218	203.684	189.881
	207.437	128.071	191.545	237.386
	216.101	132.798	166.133	248.406
	141.318	91.423	110.764	167.640
MEAN	208.804	117.785	165.698	204.202
±SEM	27.118	7.402	13.179	12.872
INGESTED UREA Iu			44.321	88.643
			45.007	90.014
			38.369	76.739
			37.825	75.739
			44.818	89.636
			39.801	79.602
PRODUCTION Pu	171.487	99.237	112.323	105.678
	183.500	133.965	120.430	97.566
	332.981	121.218	165.315	113.142
	207.437	128.071	153.720	161.736
	216.101	132.798	121.295	158.770
	141.318	91.423	70.963	88.038
MEAN	208.804	117.785	124.008	120.822
±SEM	27.118	7.402	13.613	12.933

3F Urea Hydrolysis in six adult males consuming diets with different nitrogen content (mgN/kg/d).

DIET	REF 11.2gN	LP 4.8gN	LP-U1 8.0gN	LP-U2 11.2gN
	43.371	26.453	6.159	64.917
	69.322	63.473	15.446	54.963
	168.077	39.515	100.615	115.300
	78.926	25.423	68.247	99.632
	117.585	41.115	6.247	105.324
	23.233	12.679	12.679	40.413
MEAN	83.419	34.621	35.008	80.092
±SEM	21.432	7.241	16.241	12.509

3G The fate of hydrolysed urea-nitrogen in six adult males consuming diets with different nitrogen content (mgN/kg/d).

DIET	REF 11.2gN	LP 4.8gN	LP-U1 8.0gN	LP-U2 11.2gN
UREA FORMATION Pr	14.068	6.764	8.041	7.067
	13.191	5.100	3.704	7.267
	23.396	6.050	6.415	9.931
	10.662	7.268	6.514	5.720
	8.219	5.528	3.192	11.133
	2.691	2.132	3.049	6.458
MEAN	12.038	5.974	5.153	7.962
±SEM	2.818	1.029	0.859	0.891
SYNTHETIC PATHWAYS S	29.303	19.689	-1.881	57.849
	56.131	58.373	11.762	47.696
	144.681	33.466	94.199	105.370
	68.264	18.155	61.732	93.912
	109.367	35.587	3.691	94.192
	20.543	9.614	9.631	33.955
MEAN	71.382	29.147	29.856	72.163
±SEM	19.505	7.085	15.900	12.006

3H Plasma urea levels, urea pool size, renal clearance and bowel clearance in six adult males consuming diets with different nitrogen content (mgN/kg/d).

DIET	REF 11.2gN	LP 4.8gN	LP-U1 8.0gN	LP-U2 11.2gN
PLASMA UREA mgN/100ml	16.652	16.560	20.480	29.650
	19.437	15.631	22.150	24.100
	17.488	13.775	21.580	-
	20.902	-	22.800	30.000
	15.538	20.167	21.900	31.150
	15.411	12.216	17.184	19.860
MEAN	17.565	15.670	21.016	26.952
±SEM	0.899	1.352	0.827	2.152
UREA POOL gN	7.542	7.500	9.275	13.423
	8.483	6.822	9.666	10.517
	8.307	6.543	10.251	-
	10.033	-	10.943	14.4000
	6.844	8.883	9.646	13.720
	7.170	5.683	7.995	9.239
MEAN	8.063	7.086	9.629	12.261
±SEM	0.472	0.535	0.405	1.006
RENAL CLEARANCE ml/min	38.555	22.089	36.216	21.861
	28.969	22.198	33.475	26.925
	54.577	34.491	27.686	-
	36.194	-	31.744	27.014
	31.433	24.270	36.151	22.723
	42.814	36.526	31.801	35.734
MEAN	38.757	27.915	32.962	26.851
±SEM	3.754	3.141	1.375	2.458
BOWEL CLEARANCE ml/min	13.053	8.029	1.511	10.943
	17.554	19.987	3.452	11.270
	55.625	16.681	27.013	-
	22.229	-	17.570	19.537
	37.518	10.122	1.563	16.727
	8.423	5.402	4.111	11.350
MEAN	25.734	12.042	9.203	13.965
±SEM	7.155	2.725	4.335	1.759

APPENDIX 4 Individual Data for Urea Kinetics in Six Adult Females consuming Diets with Different Nitrogen and Sulphur Amino Acid Content to Demonstrate the Influence of Sulphur Amino Acids on Urea-Nitrogen Salvage.

4A Urinary Nitrogen Excretion in six adult females consuming diets of different nitrogen and sulphur amino acid content (mgN/kg/d).

DIET EXCRETION mgN/kg/d	LP 26g P	LP-M 26g P 600mg meth	LP-U 26g P 6.9g urea	LP-UM 26g P 600mg met 6.9g urea
DAY 1	116.103	126.728	104.467	120.282
	-	84.608	105.475	125.165
	136.831	184.639	206.584	210.888
	179.473	168.259	162.216	171.771
	63.761	143.752	115.964	143.875
	149.377	206.753	166.032	156.798
MEAN	129.109	152.456	143.456	154.797
±SEM	19.296	17.865	16.897	13.695
DAY 2	83.460	88.355	110.696	121.267
	82.997	82.278	117.531	132.456
	134.955	135.249	180.373	171.608
	97.954	103.413	118.109	111.249
	102.853	65.316	99.114	146.381
	110.143	108.866	159.232	117.887
MEAN	102.060	97.246	130.843	133.475
±SEM	7.904	9.899	12.924	9.143
DAY 3	79.528	70.370	114.785	133.040
	80.714	78.822	103.104	118.777
	105.235	90.364	154.948	155.401
	80.612	81.872	110.311	134.514
	87.846	70.083	117.822	156.545
	122.720	114.208	86.832	121.360
MEAN	92.776	84.285	114.634	136.356
±SEM	7.177	6.742	9.238	6.632
Day 4	69.817	76.065	120.077	120.643
	79.478	81.043	99.640	124.336
	103.872	108.488	148.331	188.477
	69.527	63.876	110.211	136.342
	97.257	59.245	50.643	101.585
	85.630	91.444	129.419	138.450
MEAN	84.264	80.027	109.720	134.972
±SEM	5.785	7.416	13.636	11.986

DIET EXCRETION mgN/kg/d	LP 26g P	LP-M 26g P 600mg meth	LP-U 26g P 6.9g urea	LP-UM 26g P 600mg meth 6.9g urea
DAY 5	57.668	85.600	100.948	127.084
	71.839	64.725	115.487	140.241
	95.460	83.193	149.732	174.356
	80.822	65.817	124.701	128.907
	70.301	47.498	131.993	119.860
	91.176	76.449	124.077	128.195
MEAN	77.888	70.547	124.490	136.441
±SEM	5.766	5.799	6.656	8.040
AVERAGE	64.147	80.515	111.150	123.649
DAY4/5	75.913	73.428	107.035	131.758
	99.946	96.684	148.985	181.887
	74.826	64.782	116.978	132.872
	84.678	53.763	88.606	110.113
	88.218	84.446	126.926	133.664
MEAN	81.288	75.603	116.613	135.657
±SEM	5.073	6.179	8.287	9.935

4B Urinary Urea Excretion in six adult females consuming diets of different nitrogen and sulphur amino acid content (mgN/kg/d).

DIET EXCRETION mgN/kg/d	LP 26g P	LP-M 26g P 600mg meth	LP-U 26g P 6.9g urea	LP-UM 26g P 600mg meth 6.9g urea
DAY 1	127.671	111.230	96.304	127.997
	-	63.772	114.703	125.040
	105.616	189.937	210.546	172.004
	170.089	167.548	171.241	151.684
	108.825	127.199	117.982	116.099
	140.801	199.416	191.369	156.194
MEAN	130.620	143.472	150.361	141.503
±SEM	11.785	21.114	19.135	8.848
DAY 2	54.984	75.512	106.553	111.724
	52.183	50.417	136.982	126.404
	92.150	123.152	177.182	157.098
	131.654	92.570	117.725	98.922
	84.654	61.280	103.148	123.890
	95.821	81.553	168.431	160.634
MEAN	85.242	80.747	135.004	129.745
±SEM	12.004	10.432	12.940	10.042
DAY 3	97.935	65.714	105.512	141.925
	71.589	48.170	96.533	90.016
	70.000	80.523	127.349	148.309
	92.806	79.202	146.425	134.180
	74.654	56.973	153.479	126.536
	102.671	93.723	147.479	122.538
MEAN	84.942	70.718	129.463	127.251
±SEM	5.923	6.879	9.479	8.398
DAY 4	65.668	63.463	105.000	117.298
	64.559	53.965	100.546	115.907
	76.868	108.413	168.205	178.810
	61.832	61.285	104.328	91.699
	92.982	42.063	55.542	91.551
	81.419	94.424	133.289	161.269
MEAN	73.888	70.602	111.152	126.089
±SEM	4.933	10.370	15.313	14.803
DAY 5	22.453	71.581	89.938	88.960
	54.831	38.218	99.599	116.067
	62.443	100.919	151.587	127.704
	79.409	88.139	104.712	93.072
	68.464	33.102	102.184	93.494
	78.564	69.950	148.564	90.851
MEAN	61.027	67.115	116.097	101.691
±SEM	8.620	11.012	10.944	6.594

DIET EXCRETION mgN/kg/d	LP 26g P	LP-M 26g P 600mg meth	LP-U 26g P 6.9g urea	LP-UM 26g P 600mg meth 6.9g urea
AVERAGE	45.497	68.292	97.966	104.068
DAY4/5	60.016	46.613	100.069	115.987
	70.146	104.906	160.459	154.969
	70.030	73.811	104.505	92.334
	81.536	37.877	77.304	92.455
	80.083	83.005	140.417	128.414
MEAN	67.885	69.084	113.458	114.705
±SEM	5.502	9.965	12.572	9.865

4C Urinary Ammonia Excretion in six adult females consuming diets with different nitrogen and sulphur amino acid content (mgN/kg/d).

DIET EXCRETION mgN/kg/d	LP 26g P	LP-M 26g P 600mg meth	LP-U 26g P 6.9g urea	LP-UM 26g P 600mg meth 6.9g urea
DAY 1	7.205	7.748	4.084	7.065
	-	7.159	7.753	5.923
	4.447	10.835	8.727	7.808
	5.377	7.356	4.476	4.638
	5.783	8.343	8.163	4.789
	7.813	6.644	7.513	7.312
MEAN	6.125	8.014	6.786	6.256
±SEM	0.613	0.610	0.812	0.550
DAY 2	4.270	7.298	4.984	5.357
	4.510	5.843	6.693	6.260
	3.656	9.269	6.576	7.453
	5.052	4.815	4.328	4.018
	6.130	5.479	6.672	7.334
	4.992	5.125	6.077	5.125
MEAN	4.775	6.308	5.888	5.924
±SEM	0.339	0.689	0.409	0.549
DAY 3	4.115	5.807	4.736	5.559
	4.575	7.785	6.164	3.997
	3.257	5.532	5.261	4.676
	3.191	5.864	3.722	7.149
	5.904	4.699	5.105	6.521
	4.808	5.359	5.543	3.639
MEAN	4.308	5.841	5.089	5.257
±SEM	0.419	0.425	0.336	0.572
DAY 4	4.550	4.938	5.031	5.885
	4.318	5.313	8.202	5.185
	2.985	6.138	4.050	6.848
	3.191	3.486	3.722	2.349
	6.446	4.699	1.370	4.895
	3.539	6.077	4.891	3.907
MEAN	4.172	5.109	4.544	4.845
±SEM	0.520	0.404	0.908	0.641
DAY 5	3.929	6.444	5.512	6.196
	5.313	8.555	5.409	5.441
	2.944	7.056	4.843	3.883
	4.210	4.609	3.383	3.043
	6.370	3.691	6.069	4.488
	4.624	4.274	4.624	4.324
MEAN	4.565	5.817	4.973	4.563
±SEM	0.482	0.746	0.381	0.457

DIET EXCRETION mgN/kg/d	LP 26g P	LP-M 26g P 600mg meth	LP-U 26g P 6.9g urea	LP-UM 26g P 600mg meth 6.9g urea
AVERAGE	4.260	5.641	5.256	6.030
DAY4/5	4.782	6.826	6.899	5.304
	2.965	6.566	4.420	5.464
	3.666	4.010	3.560	2.673
	6.411	4.354	3.563	4.699
	4.046	5.235	4.767	4.101
MEAN	4.355	5.439	4.744	4.712
±SEM	0.480	0.465	0.510	0.489

4D Apparent Nitrogen Balance in six adult females consuming diets with different nitrogen and sulphur amino acid content (mgN/kg/d).

DIET	LP 26g P	LP-M 26g P 600mg meth	LP-U 26g P 6.9g urea	LP-UM 26g P 600mg meth 6.9g urea
INTAKE	64.596	65.528	114.286	115.217
	66.774	67.737	118.138	119.101
	86.848	88.100	153.653	154.906
	61.448	62.334	108.715	109.601
	62.651	63.554	110.843	111.747
	69.449	70.451	122.871	123.873
LOSSES	88.147	104.515	135.150	147.649
-total	99.913	97.428	131.035	155.758
urinary	123.946	120.684	172.985	205.887
N + 24	98.926	88.782	140.978	156.872
mgN/kg/d	108.678	77.763	112.606	134.113
faecal &	112.218	108.446	150.926	157.664
misc.				
losses				
BALANCE	-23.551	-38.987	-20.864	-32.432
	-33.139	-29.691	-12.897	-36.657
	-37.098	-32.584	-19.332	-50.981
	-37.378	-26.448	-32.263	-47.271
	-46.027	-14.209	-1.763	-22.366
	-42.769	-37.995	-28.055	-33.791
MEAN	-36.660	-29.986	-19.196	-37.250
±SEM	3.215	3.712	4.456	4.266

4E Urea Appearance and Production in six adult females consuming diets with different nitrogen and sulphur amino acid content (mgN/kg/d).

DIET	LP 26g P	LP-M 26g P 600mg meth	LP-U 26g P 6.9g urea	LP-UM 26g P 600mg met 6.9g urea
APPEARANCE	62.612	85.968	140.317	91.647
Au	61.667	66.084	111.870	112.103
	104.256	87.471	150.587	158.477
	95.486	91.654	129.088	126.915
	101.235	86.156	159.220	129.428
	100.551	85.152	171.659	191.112
MEAN	87.621	83.743	143.845	134.547
±SEM	8.142	3.656	8.780	14.376
INGESTED			49.689	49.689
UREA			51.364	51.364
Iu			66.806	66.806
			47.267	47.267
			48.193	48.193
			53.422	53.422
PRODUCTION	62.612	85.968	90.628	41.958
Pu	61.667	66.084	60.506	60.739
	104.256	87.471	83.781	91.671
	95.486	91.654	81.821	79.648
	101.235	86.156	111.027	81.235
	100.551	85.152	118.237	137.690
MEAN	87.621	83.743	91.000	82.157
±SEM	8.142	8.142	8.582	13.237

4F Urea Hydrolysis in six adult females consuming diets with different nitrogen and sulphur amino acid content (mgN/kg/d).

DIET	LP 26g P	LP-M 26g P 600mg meth	LP-U 26g P 6.9g urea	LP-UM 26g P 600mg meth 6.9g urea
	17.115	17.677	42.351	0.000
	1.651	19.471	11.774	0.000
	34.110	0.000	0.000	3.509
	25.456	17.843	24.583	34.581
	19.698	48.279	81.916	36.973
	20.468	2.147	31.241	62.698
MEAN	19.750	17.570	31.978	22.960
±SEM	4.373	7.055	11.673	10.556

4G The fate of hydrolysed urea-nitrogen in six adult females consuming diets with different nitrogen and sulphur amino acid content (mgN/kg/d).

DIET	LP 26g P	LP-M 26g P 600mg meth	LP-U 26g P 6.9g urea	LP-UM 26g P 600mg meth 6.9g urea
UREA FORMATION Pr	2.059 1.306 3.790 2.672 2.527 3.892	2.592 2.150 2.559 1.538 2.492 2.129	4.171 3.169 4.457 3.522 8.122 6.757	2.499 2.813 2.455 4.881 6.965 4.593
MEAN ±SEM	2.708 0.408	2.243 0.164	5.033 0.803	4.043 0.729
SYNTHETIC PATHWAYS S	15.055 0.345 30.320 22.784 17.171 16.576	15.085 17.320 0.000 16.305 45.787 0.018	38.180 8.605 0.000 21.061 73.794 24.485	0.000 0.000 1.054 29.700 30.009 58.105
MEAN ±SEM	17.040 4.051	15.753 6.843	27.688 10.678	19.811 9.670

4H Plasma urea level and urea pool size in six adult females consuming diets with different nitrogen and sulphur amino acid content (mgN/kg/d).

DIET	LP 26g P	LP-M 26g P 600mg meth	LP-U 26g P 6.9g urea	LP-UM 26g P 600mg meth 6.9g urea
PLASMA	18.889	12.725	15.450	24.059
UREA	11.135	9.942	13.630	27.638
mgN/100ml	24.258	15.708	9.375	24.854
	12.328	17.895	10.390	29.229
	20.679	22.071	8.365	34.796
	16.702	20.082	25.160	-
MEAN	17.332	16.404	13.728	28.115
±SEM	2.045	1.859	2.533	1.913
UREA	5.970	4.022	4.883	7.604
POOL	3.508	3.133	4.294	8.310
gN	6.441	4.171	2.489	6.599
	4.124	5.987	3.476	9.779
	6.657	7.106	2.693	11.202
	5.065	6.090	7.630	-
MEAN	5.294	5.085	4.244	8.699
±SEM	0.524	0.624	0.774	0.812

APPENDIX 5 **Individual Data for Urea Kinetics in six Adult Females consuming Diets with varied Nitrogen Source to Demonstrate the Influence of Glycine on Urea Salvage.**

5A Urinary Nitrogen Excretion in six adult females consuming diets of different nitrogen source (mgN/kg/d).

DIET EXCRETION mgN/kg/d	LP 26g P	LP-U 26g P 6.9g urea	LP-UG 26g P 8.6g gly 3.4g urea
DAY 1	116.103	104.467	140.113
	-	105.475	102.516
	136.831	206.584	250.948
	179.473	162.216	154.055
	63.761	115.964	195.043
	149.377	166.032	193.656
MEAN	129.109	143.456	172.722
±SEM	19.296	16.897	21.132
DAY 2	83.460	110.696	124.528
	82.997	117.531	100.772
	134.955	180.373	115.304
	97.954	118.109	132.509
	102.853	99.114	165.055
	110.143	159.232	170.426
MEAN	102.060	130.843	134.766
±SEM	7.904	12.924	11.304
DAY 3	79.528	114.785	112.138
	80.714	103.104	117.829
	105.235	154.948	165.690
	80.612	110.311	103.125
	87.846	117.822	107.124
	122.720	86.832	144.611
MEAN	92.776	114.634	125.086
±SEM	7.177	9.238	10.091
Day 4	69.817	120.077	121.532
	79.478	99.640	100.156
	103.872	148.331	154.311
	69.527	110.211	99.751
	97.257	50.643	93.242
	85.630	129.419	146.536
MEAN	84.264	109.720	119.255
±SEM	5.785	13.636	10.646

DIET EXCRETION mgN/kg/d	LP 26g P	LP-U 26g P 6.9g urea	LP-UG 26g P 8.6g gly 3.4g urea
DAY 5	57.668	100.948	114.505
	71.839	115.487	95.385
	95.460	149.732	138.602
	80.822	124.701	95.657
	70.301	131.993	94.009
	91.176	124.077	116.575
MEAN	77.888	124.490	109.122
±SEM	5.766	6.656	7.193
AVERAGE DAY4/5	64.147	111.150	118.253
	75.913	107.035	97.930
	99.946	148.985	146.980
	74.826	116.978	97.840
	84.678	88.606	93.600
	88.218	126.926	132.554
MEAN	81.288	116.613	114.526
±SEM	5.073	8.287	8.914

5B Urinary Urea Excretion in six adult females consuming diets of different nitrogen source (mgN/kg/d).

DIET EXCRETION mgN/kg/d	LP 26g P	LP-U 26g P 6.9g urea	LP-UG 26g P 8.6g gly 3.4g urea
DAY 1	127.671	96.304	130.062
	-	114.703	95.955
	105.616	210.546	225.887
	170.089	171.241	70.990
	108.825	117.982	273.886
	140.801	191.369	207.529
MEAN	130.620	150.361	167.385
±SEM	11.785	19.135	32.743
DAY 2	54.984	106.553	112.857
	52.183	136.982	85.875
	92.150	177.182	110.668
	131.654	117.725	106.588
	84.654	103.148	137.831
	95.821	168.431	124.174
MEAN	85.242	135.004	112.999
±SEM	12.004	12.940	7.127
DAY 3	97.935	105.512	102.702
	71.589	96.533	90.080
	70.000	127.349	183.967
	92.806	146.425	79.084
	74.654	153.479	110.708
	102.671	147.479	124.140
MEAN	84.942	129.463	115.114
±SEM	5.923	9.479	15.188
DAY 4	65.668	105.000	118.183
	64.559	100.546	77.512
	76.868	168.205	144.760
	61.832	104.328	90.739
	92.982	55.542	76.521
	81.419	133.289	142.087
MEAN	73.888	111.152	108.300
±SEM	4.933	15.313	12.695
DAY 5	22.453	89.938	100.202
	54.831	99.599	76.035
	62.443	151.587	123.862
	79.409	104.712	104.771
	68.464	102.184	113.584
	78.564	148.564	119.766
MEAN	61.027	116.097	106.370
±SEM	8.620	10.944	7.067

DIET EXCRETION mgN/kg/d	LP 26g P	LP-U 26g P 6.9g urea	LP-UG 26g P 8.6g gly 3.4g urea
AVERAGE DAY4/5	45.497 60.016 70.146 70.030 81.536 80.083	97.966 100.069 160.459 104.505 77.304 140.417	109.798 76.822 135.010 97.282 93.419 131.669
MEAN ±SEM	67.885 5.502	113.458 12.572	107.333 9.291

5C Urinary Ammonia Excretion in six adult females consuming diets with different nitrogen source (mgN/kg/d).

DIET EXCRETION mgN/kg/d	LP 26g P	LP-U 26g P 6.9g urea	LP-UG 26g P 8.6g gly 3.4g urea
DAY 1	7.205	4.084	6.040
	-	7.753	8.668
	4.447	8.727	9.520
	5.377	4.476	3.501
	5.783	8.163	15.196
	7.813	7.513	5.960
MEAN	6.125	6.786	4.148
±SEM	0.613	0.812	1.660
DAY 2	4.270	4.984	5.559
	4.510	6.693	6.067
	3.656	6.576	4.739
	5.052	4.328	7.134
	6.130	6.672	6.114
	4.992	6.077	4.825
MEAN	4.775	5.888	5.740
±SEM	0.339	0.409	0.368
DAY 3	4.115	4.736	4.829
	4.575	6.164	6.597
	3.257	5.261	5.908
	3.191	3.722	3.737
	5.904	5.105	5.181
	4.808	5.543	4.407
MEAN	4.308	5.089	5.110
±SEM	0.419	0.336	0.421
DAY 4	4.550	5.031	5.512
	4.318	8.202	7.079
	2.985	4.050	5.678
	3.191	3.722	5.894
	6.446	1.370	3.404
	3.539	4.891	4.040
MEAN	4.172	4.544	5.268
±SEM	0.520	0.908	0.544
DAY 5	3.929	5.512	4.612
	5.313	5.409	6.404
	2.944	4.843	4.781
	4.210	3.383	7.194
	6.370	6.069	5.979
	4.624	4.624	4.190
MEAN	4.565	4.973	5.527
±SEM	0.482	0.381	0.481

DIET EXCRETION mgN/kg/d	LP 26g P	LP-U 26g P 6.9g urea	LP-UG 26g P 8.6g gly 3.4g urea
AVERAGE	4.260	5.256	5.092
DAY4/5	4.782	6.899	6.758
	2.965	4.420	5.261
	3.666	3.560	6.500
	6.411	3.563	4.605
	4.046	4.767	4.110
MEAN	4.355	4.744	5.388
±SEM	0.480	0.510	0.427

5D Apparent Nitrogen Balance in six adult females consuming diets with different nitrogen source (mgN/kg/d).

DIET	LP 26g P	LP-U 26g P 6.9g urea	LP-UM 26g P 8.6g gly 3.4g urea
INTAKE	64.596	114.286	114.286
	66.774	118.138	118.138
	86.848	153.653	153.653
	61.448	108.715	108.715
	62.651	110.843	110.843
	69.449	122.871	122.871
LOSSES	88.147	135.150	142.253
-total	99.913	131.035	121.930
urinary	123.946	172.985	170.980
N + 24	98.926	140.978	121.840
mgN/kg/d	108.678	112.606	117.600
faecal &	112.218	150.926	156.554
misc. losses			
BALANCE	-23.551	-20.864	-27.967
	-33.139	-12.897	-3.792
	-37.098	-19.332	-17.327
	-37.378	-32.263	-13.125
	-46.027	-1.763	-6.757
	-42.769	-28.055	-33.683
MEAN	-36.660	-19.196	-17.109
±SEM	3.215	4.456	4.806

5E Urea Appearance and Production in six adult females consuming diets with different nitrogen source (mgN/kg/d).

DIET	LP 26g P	LP-U 26g P 6.9g urea	LP-UG 26g P 8.6g gly 3.4g urea
APPEARANCE	62.612	140.317	112.566
Au	61.667	111.870	108.445
	104.256	150.587	159.282
	95.486	129.088	95.282
	101.235	159.220	126.019
	100.551	171.659	164.002
MEAN	87.621	143.845	127.669
±SEM	8.142	8.780	11.463
INGESTED		49.689	24.845
UREA		51.364	25.682
Iu		66.806	33.403
		47.267	23.634
		48.193	24.096
		53.422	26.711
PRODUCTION	62.612	90.628	87.721
Pu	61.667	60.506	82.763
	104.256	83.781	125.879
	95.486	81.821	72.055
	101.235	111.027	101.923
	100.551	118.237	137.291
MEAN	87.621	91.000	101.272
±SEM	8.142	8.582	10.461

5F Urea Hydrolysis in six adult females consuming diets with different nitrogen source (mgN/kg/d).

DIET	LP 26g P	LP-U 26g P 6.9g urea	LP-UG 26g P 8.6g gly 3.4g urea
	17.115	42.351	2.768
	1.651	11.774	31.623
	34.110	0.000	24.272
	25.456	24.583	0.000
	19.698	81.916	32.600
	20.468	31.241	32.333
MEAN	19.750	31.978	20.599
±SEM	4.373	11.673	6.216

5G The fate of hydrolysed urea-nitrogen in six adult females consuming diets with different nitrogen source (mgN/kg/d).

DIET	LP 26g P	LP-U 26g P 6.9g urea	LP-UG 26g P 8.6g gly 3.4g urea
UREA FORMATION Pr	2.059 1.306 3.790 2.672 2.527 3.892	4.171 3.169 4.457 3.522 8.122 6.757	4.742 4.078 7.541 3.774 4.974 7.319
MEAN ±SEM	2.708 0.408	5.033 0.803	5.405 0.665
SYNTHETIC PATHWAYS S	15.055 0.345 30.320 22.784 17.171 16.576	38.180 8.605 0.000 21.061 73.794 24.485	0.000 27.545 16.730 0.000 27.627 25.014
MEAN ±SEM	17.040 4.051	27.688 10.678	16.153 5.361

5H Plasma urea level and urea pool size in six adult females consuming diets with different nitrogen source (mgN/kg/d).

DIET	LP 26g P	LP-U 26g P 6.9g urea	LP-UG 26g P 8.6g gly 3.4g urea
PLASMA	18.889	15.450	19.290
UREA	11.135	13.630	15.245
mgN/100ml	24.258	9.375	17.875
	12.328	10.390	19.700
	20.679	8.365	22.935
	16.702	25.160	37.305
MEAN	17.332	13.728	22.058
±SEM	2.045	2.533	3.217
UREA	5.970	4.883	6.096
POOL	3.508	4.294	4.803
gN	6.441	2.489	4.746
	4.124	3.476	6.591
	6.657	2.693	7.384
	5.065	7.630	11.312
MEAN	5.294	4.244	6.822
±SEM	0.524	0.774	0.991

APPENDIX 6 **Individual Data for Inorganic Sulphate Excretion in Male and Female Subjects consuming Diets with varied Nitrogen and Sulphur Amino Acid Content.**

6A Urinary inorganic sulphate excretion in six adult males consuming diets with varied nitrogen content (mmol/day).

DIET EXCRETION mmol/day	REF 70g P	LP 30g P	LP-U1 30g P 6.9g urea	LP-U2 30g P 13.7g urea
	22.569	21.706	23.757	22.946
	27.577	18.983	22.641	18.152
	26.145	19.550	25.901	27.225
	19.256	18.430	25.767	24.458
	26.254	17.781	23.398	13.048
	15.595	21.103	18.800	25.992
mean	22.899	19.592	23.377	21.975
±sem	1.918	0.629	1.060	2.199

6B Urinary inorganic sulphate excretion in six adult females consuming diets of varied nitrogen and sulphur amino acid content (mmol/day with mean ±sem indicated).

LP 26g P	LP-M 26g P 600mg meth	LP-U 26g P 6.9g urea	LP-UM 26g P 600mg meth 6.9g urea	LP-UG 26g P 8.6g gly 3.4g urea
14.271	23.498	13.422	17.488	14.009
20.566	20.310	14.950	22.083	11.931
7.962	23.846	14.865	16.491	15.053
18.818	25.184	13.867	20.248	12.707
10.384	11.832	14.077	19.249	11.553
15.258	19.113	12.983	19.003	17.905
14.453	20.631	14.027	19.094	13.860
1.964	1.993	0.318	0.809	0.969

APPENDIX 7 **Individual Data for 5-oxoproline Excretion in Male and Female Subjects consuming Diets with varied Nitrogen and Sulphur Amino Acid Content.**

7A Urinary 5-oxoproline excretion in six adult males consuming diets with varied nitrogen content (µmol/day).

DIET EXCRETION µmol/day	REF 70g P	LP 30g P	LP-U1 30g P 6.9g urea	LP-U2 30g P 13.7g urea
	155.46	225.34	365.22	256.33
	268.46	682.25	329.00	283.56
	254.98	811.79	518.69	311.63
	283.91	663.43	467.61	333.93
	237.74	366.98	307.00	238.42
	142.13	314.50	370.02	242.55
mean	223.78	510.71	392.92	277.74
±sem	24.57	97.30	33.74	15.90

7B Urinary 5-oxoproline excretion in six adult females consuming diets of varied nitrogen and sulphur amino acid content (µmol/day with mean ±sem indicated).

LP 26g P	LP-M 26g P 600mg meth	LP-U 26g P 6.9g urea	LP-UM 26g P 600mg meth 6.9g urea	LP-UG 26g P 8.6g gly 3.4g urea
442	487	106	556	131
400	284	345	497	250
266	409	209	342	170
498	648	212	454	110
280	182	119	212	165
421	336	287	454	289
385	391	213	419	186
38	67	38	50	28

REFERENCES

Alleyne GAO, McFarlane-Anderson N, Lupianez JA, Hohelano P, Sanchez-Medina F. Metabolism of Ammonia. In:Nitrogen Metabolism in Man. Eds Waterlow JC & Stephens JML. Appl. Sci. Pub,London. (1981) p169-184

Allison JB, Bird JWC. Elimination of Nitrogen from the body. In: Mammalian Protein Metabolism, Volume 1. Ed Munro H.N, Allison J.B. Acad Press NY (1964), p483-512,

Anderson HL, Heindel MB, Linkswiler H. Effect of nitrogen balance of adult man of varying nitrogen source and level of calorie intake. J. Nutr (1969), **99**, 82-90

Aoyagi T, Engstrom GW, Evans WB, Summerskill WHJ. Gastrointestinal urease in man. I Activity of mucosal urease. Gut (1966), **7**, 631-635

Atinmo T, Mbofung CMF, Egun G, Osotimetin B. Nitrogen balance studies in young nigerian adult males using four levels of protein intake. Br. J. Nutr (1988), **60**, 451-458

Atkinson DE, Bourke E. The role of ureagenesis in pH homeostasis. TIBS (1984), **9**, 297-300

Bender DA. Amino Acid Metabolism (1985), Wiley Interscience Publishers

Benevenga, NJ & Harper AE. Alleviation of methionine and homocystine toxicity in the rat. J. Nutr (1967), **93**, 44-52

Bessman SP. The justification theory: the essential nature of the non-essential amino acids. Nut Rev (1979), **37**, 209-220

Bodwell CE, Scuster EM, Brooks B, Womack M, Steele P, Ahrens R. Obligatory urinary and faecal nitrogen losses in young women, older men and young men and the factorial estimation of adult human protein requirements. Am. J. Clin. Nutr (1979), **32**, 2450-2459

Brenner BM, Meyer TW, Hostetter TH. Dietary protein intake and the progressive nature of kidney disease. New Eng. J. Med (1982), **307**, 652-659

Bressani R, Braham JE, Elias LG, Balconi R. Urinary nitrogen and sulfur excretion in dogs under different dietary treatment. J. Nutr (1965), **87**, 77-84

Brockenbank JT, Wolfe S. Dietary treatment of renal insufficiency. Arch. Dis. Child (1993), **69**, 704-708

Brown CL, Hill MJ, Richards P. Bacterial ureases in ureamic man. Lancet, 21 Aug 1971, 406-408

Brown JH & Allison JB. Effects of excess dietary DL-methionine and/or L-arginine on rats. Proc Soc Exp Biol Med (1948), **69**, 196-200

Bundy R, Persaud C, Jackson AA. Measurement of urea kinetics with a single dose of [15N15N]-urea in free living female vegetarians on their habitual diet. Int. J. Food. Sci. Nut (1994)

Bundy R, Persaud C, Murphy J,Hounslow A, Wootton SA, Jackson AA. Metabolism of lactose-ureide labelled with ^{15}N and ^{13}C in humans. Proc Nut Soc (1995), in press

Calloway DH, Spector H. Nitorgen balance as related to caloric and protein intake in active young men. Am. J. Clin. Nut (1954), **2**, 405-412

Calloway DH, Odell ACF, Margen S. Sweat and miscellaneous nitrogen losses in human balance studies. J Nutr (1971), **101**, 775-786

Calloway D.H & Margen S. Variation in endogenous nitrogen excretion and dietary nitrogen utilisation as determinants of human protein requirement. J. Nutr (1971), **101**, 205-216

Calloway DH. Nitrogen balance of men with marginal intakes of protein and energy. J Nutr (1975), **105** 914-923

Carraro F, Kimbrough TD, Wolfe RR. Urea kinetics in humans at two levels of exercise intensity. J Appl Phys (1993), **75**, 1180-1185

Chadwick VS, Jones JD, Debongie JC. Urea, urinc acid and creatine fluxes through the small intestine in man. Gut (1977), **18**, A944

Chen SC, Fox HM, Kies C. Nitrogenous factors affecting the adequacy of rice to meet the protein requirements of human adult. J Nutr (1967), **92**, 429-434

Child S. Fourth year medical student report, University of Southampton 1995

Clarke HE, Yess NJ, Vermillon EJ, Goodwin AF, Mertz ET. Effect of certain factors on nitrogen retention and lysine requirements of adult human subjects III source of supplementary nitrogen. J. Nutr (1963a), **79**, 131-139

Clarke HE, Kenney MA, Goodwin AF, Goyal K, Mertz ET. Effect of certain factors on nitrogen retention and lysine requirements of adult human subjects IV total nitrogen intake. J Nutr (1963b), **81**, 223-229

Clarke HE, Woodward L. Influence of variable quantities of methionine on nitrogen retention of adult human subjects. Am. J. Clin. Nut (1966), **18**, 100-104

Clarke HE, How JM, Shannon BM, Carlson K, Kolski SM. Requirements of adult human subjects for methionine and cystine. Am. J. Clin. Nut (1970), **23**, 731-738

Clugston GA, Garlick PJ. The response of protein and energy metabolism to food intake in lean and obese man. Hum. Nutr. Clin. Nutr (1982), **36c**, 57-70

Cohen HP, Choitz HC, Berg CP. Response of rats to diets high in methionine and related compounds. J Nutr (1958), **64**, 554-569

Cohen P.P. Regulation of the ornithine-urea cycle enzymes In: Nitrogen Metabolism in Man Ed Waterlow J.C. & Stephen J.M.L. Appl Sci Pub,London. (1981), p215-228

Cox, Mueller, Elman. Nitrogen retention studies in rats, dogs and man. J Nutr (1947), **33**, 437

Cummings JH, MacFarlane GT. The control and consequences of bacterial fermentation in the human colon. J App Bact (1991), **70**, p443-459

Danielsen M, Jackson AA. Limits of adaptation to a diet low in protein in normal man: urea kinetics. Clin Sci (1992), **83**, p103-108

Darling P, Wykes LJ, Clarke R, Papageorgiou A, Pencharz PB. Utilisation of non-protein nitrogen in whey-dominant formulae by low birthweight infants. Clin Sci (1993), **84**, 543-548

Das TK, Waterlow JC. The rate of adaptation of urea cycle enzymes, aminotransferases and glutamate dehydrogenase to chabges in dietary protein intake. Br J Nutr (1974), **32**, 353-373

deBenoist B, Jackson AA, Hall JS, Persaud C. Whole body protein turnover in Jamaican women during pregancy. Hum Nutr Clin Nutr (1985), **39C**, 167-179

Doherty J, Jackson AA. The effect of dietary pectin on rapid catch-up weight gain and urea kinetics on children recovering from severe undernutrition. Acta Paed (1992), **81**, 514-517

Durnin JVGA, Rahaman MM. The assessment of the amount of fat in the human body from measurements of skinfold thickness Br J Nutr (1967), **21**, 681-689

Eckert JN. Sulphur balance indexes of casein in adult dogs with or without addition of methionine. Arch Biochem Biophys (1948), **19**, 379-387

El-Khouri AE, Fukagawa NK, Sanchez M, Tsay RH, Gleason RE, Chapman TE, Young VR. Validation of the tracer balance concept with reference to leucine: 24 hour intravenous tracer studies with L-[1-13C]leucine and [15N15N]urea. Am. J. Clin. Nutr (1994a), **59**, 1000-1011

El-Khouri AE, Fukagawa NK, Sanchez M, Tsay RH, Gleason RE, Chapman TE, Young VR. The 24 hour pattern and rate of leucine oxidation, with particular reference to tracer estimates of leucine requirements in healthy adults. Am J Clin Nutr (1994b), **59**, 1012-1020

Fei YJ, Kanai Y, Nussberger S, Ganapathy V, Laibach FH, Romero MF, Singh SK, Boron WF, Heidiger MA. Expression cloning of a mammalian proton-coupled oligopeptide transporter Nature (1994), **368**, 563-566

Fern EB, Garlick PJ, McNurlan MA, Waterlow JC. The excretion of isotope in urea and ammonia for estimating protein turnover in man with [15N]glycine. Clin Sci (1981), **61** 217-228

Finkelstein JD, Mudd SH. Transsulfuration in mammals- the methionine sparing effect of cystine. J Biol Chem (1967), **242**, 873-880

Fleck A, Munro HN. The determination of organic nitrogen in biological materials. Clinic Chimica Acta (1965), **11**, 2-12

Folin O. Laws governing the chemical composition of urine. Am J Phys (1905), **13**, 66-115

Fomon SJ, DeMaeyer EM, Owen GM. Urinary and faecal excretion of endogenous nitrogen by infants and children J Nutr (1965), **85**, 235-246

Fomon SJ, Ziegler EE, Filer LJ, Nelson SE, Edwards BB. Methionine fortification of a soy protein formula fed to infants J. Nutr (1979), **32**, 2460-2471

Forrester T, Badaloo AV, Persaud C, Jackson AA. Urea production and salvage during pregnancy in normal Jamaican women. Am J Clin Nutr (1994), **60**, 341-346

Gallina DL, Dominquez JM. Human utilisation of urea nitrogen in low calorie diets. J Nutr (1971), **101**, 1029-1036

Garlick PJ, Millward DJ, James WPT. The diurnal response of muscle and liver protein synthesis in vivo in meal fed rats. Biochem J (1973), **136**, 935-946

Garlick PJ, Glugston GA, Swick RW, Waterlow JC. Diurnal patterns of protein and energy metabolism in man. Am J Clin Nutr (1980), **33**, 1983-1986

Garza C, Scrimshaw NS, Young VR. Human protein requirements: interrelationships between energy intake and nitrogen balance in young men consuming the 1973 FAO/WHO safe level of egg protein, with added non-essential amino acids. J Nutr (1978), **108**, 90-96

Gattas V, Barrera GA, Riumallo JS, Uauy R. Protein:energy requirements of prepubertal school aged boys determined by the use of the nitrogen balance response to a mixed protein intake. Am J Clin Nutr (1990), **52**, 1037-1042

Gersovitz M, Bier D, Matthews D, Udall J, Munro HN, Young VR. Dynamic aspects of whole body glycine metabolism: influence of protein intake in young adult and elderly men
Metabolism (1980), **29**, 1087-1094

Gibson JA, Park NJ, Sladen GE, Dawson AM. The role of the colon in urea metabolism in man. Clin Sci & Mol Med (1976), **50**, 51-59

Gillin AG, Sands JM. Urea transport in the kidney Semin Nephrol (1993), **13**, 146-154

Giordano C. Use of exogenous and endogenous urea for protein synthesis in normal and uraemic subjects. J. Lab & Clin Med (1963), **62**, 231-246

Giordano C, de Pascale C, Balestrieri C, Cittadini D, Crescenzi A. Incorporation of urea ^{15}N in amino acids of patients with chronic renal failure on low protein nitrogen diet. Am J Clin Nutr (1968), **21**, 394-404

Giovannetti S, Maggoire Q. A low nitrogen diet with proteins of high biological value for severe uraemia. Lancet, 9 May 1964, 1000-1003

Gopolan C, Narasinga Rao BS. Effect of protein depletion on urinary nitorgen excretion in undernourished subjects. J Nutr (1966), **90**, 213-218

Graham DY. C. Pylori detected noninvasively by the 13C urea breath test. Lancet, 23 May 1987, p1174-1177

Gregory J, Foster K, Tyler H, Wiseman M
The Dietary and Nutritional Survey of British Adults, HMSO, London 1990

Grove G, Jackson AA. Whole body protein turnover in healthy young women at different stages of the menstrual cycle. Proc Nut Soc (1992), **51**, 44A

Grove G.
Ph. D Thesis, Southampton University 1993

Harper AE. Diet and plasma amino acids. Am J Clin Nutr (1968), **21**, 358-366

Harper AE. Dispensable and indispensable amino acid interrelationships. IN: Amino Acids- metabolism and medical applications Eds Blackburn GL, Grant JP, Young VR. Joh Wright PSG Inc. (1983)

Haussinger D, Gerok W, Sies H. Hepatic role in pH regulation. TIBS (1984), **9**, 300-302

Hausiinger D. Organisation of hepatic nitorgen metabolism and its relation to acid base homeostasis. Klin Wochenschr (1990), **68**, 1096-1101

Hegsted D.M. Balance Studies. J Nutr (1976), **106**, p307-311

Heine W, Wutzke K.D, Richter I, Walther F, Plath C. Evidence for colonic absorption of protein nitrogen in infants. Acta Paed Scand (1987), **76**, p741-744

Heine W, Mohr C, Wutzke K.D. Correlations between the intestinal microflora and whole body protein metabolism in infants In: Stable Isotopes in Paedeactric Nutrition & Metabolic Research (1990), p227

Henry CKJ. Quantitative realtionships between protein and energy metabolism: influence of body composition In: protein: energy interations Eds Scrimshaw & Schurch IDECG 1991

Hibbert J.M. & Jackson A.A. Variation in measures of urea kinetics over four years in a single adult. Eur J Clin Nutr (1991), **45**, p347-351

Hibbert J.M, Forrester T, Jackson A.A. Urea kinetics: comparison of oral and intravenous dose regimens. Eur J Clin Nutr (1992), **46**, p405-409

Hill MJ, Drasar BS. The normal colonic bacterial flora. Gut (1975), **16**, 318-323

Hiramatsu T, Cortiella J, Marchini JS, Chapman TE, Young VR. Source and amount of dietary non-specific nitrogen in relation to whole body leucine, phenylalanine and tyrosine kinetics in young men. Am. J. Clin. Nutr (1994), **59**, 1347-1355

Hoffer LJ, Yang RD, Matthews DE, Bistrian BR, Bier DM, Young VR. Effects of meal consumption on whole body leucine and alanine linetics in young adult men. Br J Nutr (1985), **53**, 31-38

Holt L, Snyderman SE. Nutr Abs Rev (1965), **35**, 1-13

Hou J et al, Effect of low protein diets on whole body nitrogen metabolism in patients with chronic renal failure. Chin. Med J (1985), **98**, 765-770

Houndslow A. MsC project, University of Portsmouth 1995

Hsia YE. Inherited hyperammononemic syndromes. Gastroent (1974), **67**, 347-374

Huang PC, Young VR, Cholakos B, Scrimshaw NS. Determination of the minimum dietary essential amino acid-to-total nitrogen ratio for beef protein fed to young men. J Nutr (1966), **90**, 416-422

Huang PC, Chong HE, Rand WM. Obligatory urinary and faecal nitrogen losses in young chinese men. J Nutr (1972), **102**, 1605-1614

Huang PC, Lin CP, Hsu JY. Protein requirements of normal infants at the age of about one year: maintenance nitrogen requirements and obligatory nitrogen losses. J Nutr (1980), **110**, 1727-1735

Ihle BU, Becker GJ, Whitworth JA, Charlwood RA, Kincaid-Smith PS. The effect of protein restriction on the progression of renal insufficiency. New Eng. J. Med (1989), **321**, 1773-1777

Inoue G, Fujita Y, Niiyama Y. Studies on protein requirements of young men fed egg protein and rice protein with excess and maintenance energy intakes. J Nutr (1973), **103**, 1673-1687

Iyengar AK, Narasinga Rao BS, Reddy V. Effect of varying protein and energy intakes on nitrogen balance in indian preschool children. Br. J. Nutr (1979), **42**, 417-423

Jackson AA, Golden MH, Jahoor PF, Landman JP. The isolation of urea nitrogen and ammonia nitrogen from biological samples for mass spectrometry. Anal Biochem (1980), **105**, 14-17

Jackson AA, Landman JP, Picou D. Urea production rates in man in relation to the dietary intake of nitrogen and the metabolic states of the individual. In: Nitrogen Metabolism in Man, Eds Waterlow J.C & Stephen J.M.L, Appl Sci Pub, London. (1981a), 247-251

Jackson AA, Shaw JCL, Barber A, Golden MHN. Nitrogen metabolism in preterm infants fed human donar breast milk: the possible essentiality of glycine. Pediatr Res (1981b), **15**, 1454-1461

Jackson A.A. Amino acids: essential and non-essential? Lancet, 7 May 1983, 1034-1038

Jackson A.A, Picou D, Landman J.P. The non-invasive measurement of urea kinetics in normal man by a constant infusion of 15N15N urea. Hum Nutr Clin Nutr (1984), **38C**, 339-354

Jackson AA, Badaloo AV, Forrester T, Hibbert JM, Persaud C. Urinary 5-oxoproline (pyroglutamic aciduria) as an index of glycine insufficiency in normal man. Br J Nutr (1987), **58**, 207-214

Jackson AA, Lamdman JP, Stevens MCG, Serjeant GR. Urea kinetics in aults with homozygous sickle cell diease. Eur J Clin Nutr (1988), **42**, 491-496

Jackson A.A. Optising amino acid and protein supply and utilisation in the new born. Proc Nutr Soc (1989), **48**, 293-301

Jackson A.A, Doherty J, de Benoist M.H, Hibbert J, Persaud C. The effect of the level of dietary protein, carbohydrate and fat on urea kinetics in young children during rapid catch-up weight gain. Br J Nutr (1990a), **64**, 371-385

Jackson AA. Protein requirements for catch-up growth. Proc Nut Soc (1990b), **49**, 507-516

Jackson AA, Persaud C, Evans N, Hall M, Rutter N, Smith S. Glycine insufficiency during infancy assessed by urinary 5-oxoproline excretion. Ear Hum Dev (1990c), **22**, 171(Abstract)

Jackson AA, Wootton SA. The energy requirements of growth and catch-up growth. In: Acitivity, energy expenditure and energy requirements of Infants and children, IDECG (1990), 185-214

Jackson AA. The glycine story. Eur. J. Clin. Nutr (1991a), 59-65

Jackson AA. Critique of protein-energy interactions in vivo: urea kinetics. In: Protein-energy interactions. Eds Scrimshaw NS, Schurch B. IDECG (1991b), 63-79

Jackson A.A. Protein metabolism in man. In: The contribution of nutrition to human and animal health. Eds Widdowson EM & Mathers ED, Camridge University Press, (1992), 92

Jackson AA, Moran BJ. Urea kinetics and TPN. Am J Clin Nutr (1992), **55**, 481

Jackson A.A. Chronic malnutrition: protein metabolism. Proc Nut Soc (1993a), **52**, 1-10

Jackson AA, Forrester T. Humn requirements for protein, amino acids and nitrogen: the role of urea salvage in adaptation to low intake. Nestle foundation annual report (1993b)

Jackson A.A, Danielsen D, Boyes S. A non-invasive method for measuring urea kinetics with a single dose of 15N15N urea in free living people. J. Nutr (1993c), **123**, 2129-2136

Jackson AA. Urea as a nutrient: bioavaiability and role in nitrogen economy. Arch. Dis. Child (1994a), **70**, 3-4

Jackson AA. (& Young VR 1994) Is leucine produced by the colonic micrflora? Am J Clin Nutr (1994b), **60**, 977-979

Jackson AA. Salvage of urea nitrogen and protein requirements. Proc Nut Soc (1995), **54**, 535-547

Jackson MJ, Beaunder AL, O'Brien WE. Mammaliam urea cycle enzymes. Ann. Rev. genet (1986), **20**, 431-464

Jahoor F. & Wolfe R.R. Reassessment of primed constant infusion tracer method to measure urea kientics. Am J Phys (1987), **252**, E557-E564

Jahoor F, Jackson A.A, Golden M.H.N. In vivo metabolism of nitrogen precursors for urea synthesis in the postprandial rat. Anal Nutr and Metab (1988), **32**, 240-244

Johnson. Effect of methionine supplementation on urinary nitrogen. J Nutr (1947), **33**, 371

Jones E.A, Smallwood R.A, Craigie A, Rosenoer V.M. The enterohepatic circulation of urea nitrogen. Clin Sci (1969), **37**, 825-836

Jourdan M, Glock C, Margens S, Bradfield RB. Sulphate, acid base and mineral balances of obese women during weight loss. Am J Clin Nutr (1980), **33**, 236-243

Kaneko K, Nishida K, Yatsuda J. Effect of methionine supplementation of a soy protein isolate on short term nitrogen balance in young women. J Nutr Sci Vitaminol (1986), **32**, 123-130

Kaplan A. Urea nitrogen and urinary ammonia. In: Standard methods of clinical chemistry. Ed: Meites S. NY Acad Press (1965), vol 5, 245-256

Kennedy N, Badaloo AV, Jackson AA. Adaptation to a marginal intake of energy in young children. Br J Nutr (1990), **63**, 145-154

Kies C, Williams E, Fox HM. Determination of first limiting nitrogenous factor in corn protein for nitrogen retention in human adults. J Nutr (1963), **86**, 350-356

Kies C. & Linkswiter H.M. Effect on nitrogen retention of men of altering the intake of essential amino acids with total nitrogen held constant. J Nutr (1965a), **85**, 136-144

Kies C, Shortridge L, Reynolds M.S. Effect on nitrogen retention of men of varying the total dietary nitrogen with essential amino acid intake kept constant. J Nutr (1965b), **85**, 260-268

Kies C, Williams E, Fox HM. Effect of 'non-specific' nitrogen intake on adequacy of cereal proteins for nitrogen retention in human adults J. Nutr (1965c), **86**, 350-356

Kies C, Fox HM, Williams ER. Effect of non-specific nitrogen supplementation on minimum corn protein requriement and first limiting amino acid for adult men J. Nutr (1967a), **92**, 377-383

Kies C, Fox HM Effect of level of total nitrogen intake on second limiting amino acid in corn for humans J. Nutr (1970a), **100**, 1275-1286

Kies C, Fox H. Urea as a source of non-specific nitrogen for humans. Proc Midwest Reg Meet Am Chem Soc (1970b)

Kies C, Fox HM. Protein nutritional value of opaque-2-corn grain in human adults. J Nutr (1972a), **102**, 757-766

Kies C. Non-specific nitrogen in the nutrition of human beings. Fed Proc (1972b), **31**, 1172-1177

Kies C, Fox HM. Comparison of urea and diammonium citrate as sources of nonspecific nitrogen for human adults. J. Nutr (1973), **103**, 664-669

Kies C. Comparative value of various sources of nonspecific nitrogen for the human. J. Ad. Fd. Chem (1974), **22**, 190-193

Kies C, Fox HM, Aprahamian S. comparative value of L, DL and D-methionine supplementation of an oat based diet for humans J. Nutr (1975), **105**, 809-814

Kies C. & Fox H. Urea as a dietary supplement for humans.Adv Exp Med Biol (1978), **105**, p103-118

Klain GJ, Vaughan DA, Vaughan LN. Some metabolic effects of methionine toxicity in the rat. J Nutr (1963), **80**, 337-341

Koishi H, Okuda T, Miyoshi H. Nutritive effects of L-methionine supplementation to the soy protein isolate in human body. Nutr. Sci. Soy Prot (Japan) (1984), **5**, 99-103

Koishi H. Nutritional adaptation of PNG highlanders. Eur J Clin Nutr (1990), **44**, 853-885

Kopple JD, Swenseid ME. Evidence that histidine is an essential amino acid in normal and chronically uraemic man. J. Clin Invest (1975), **55**, 881-891

Kopple JD. Nutritional management of chronic renal failure. Postgrad Med (1978), **64**, 135-144

Korsland MK, Kies C, Fox HM. Comparison of the protein nutritional value of TVP, methionine enriched TVP and beef for adolescent boys. J. Food Sci (1973), **38**, 637-638

Korsland MK, Kies C, Fox HM. Protein nutrition value of urea supplementation of opaque-2-corn for adolescent boys. Am. J. Clin. Nutr (1977), **30**, 371-374

Laidlaw SA, Kopple JD. Newer concepts of the indispensable amino acids. Am J Clin Nutr (1987), **46**, 593-605

Langran M, Moran B.J, Murphy J.L, Jackson A.A. Adaptation to a diet low in protein: effect of complex carbohydrate upon urea kinetics in normal man. Clin Sci (1992), **82**, 191-198

Levitan R, Biilich Co. The permeability of the human colon. Clin Res (1968), **16**, 287

Long C.L, Jeevanandam M, Kinney J.M. Metabolism and recycling of urea in man. Am J Clin Nutr (1978), **31**, 1367-1382

Lund P, Wiggins D. The ornithine requirement of urea synthesis. Biochem J (1986), **239**, 773-776

Lundquist P, Martensson J, Sorbo B, Ohman S. Turbimetry of inorganic sulphate, ester sulphate and total sulphate in urine. Clin. Chem (1980), **28**, 1178-1181

MacFarlane GT, Allison C. Utilisaiton of protein by human gut bacteria. FEMS Micro Ecol (1986), **38**, 19-24

Marchini JS, Cortiella J, Hiramatsu T, Chapman TE, Young VR. Requirements for indispensable amino acids in human: longer term amino acid kinetic study with support for the adequacy of the MIT amino acid requirement pattern. Am. J. Clin. Nutr (1993),

Matthews DE, Downey RS. Measuremetn of urea kinetics in humans: a validation of stable isotope methods. Am J Phys (1984), **246**, E519-E527

McCance & Widdowson E.M.The composition of Foods. RSC. & MAFF, 5th Edition (1991)

McClelland ISM. PhD Thesis, University of Southampton 1994

McGiven JD, Chappell JB. On the metabolic function of glutamine dehydrogenase in rat liver. FEBS Letts (1975), **52**, 1-7

Meijer A.J, Lamers W.H, Chamuleau R.A.F.M. Nitrogen metabolism and ornithine cycle function Phys Rev (1990), **70**, 701-748

Millward DJ, Nnanyelugo DO, James WPT, Garlick PJ. Protein metabolism in skeletal muscle: the effect of feeding and fasting on muscle RNA, free amino acids and plasma concentrations. Br. J. Nutr (1974), **32**,127-142

Millward D.J. & Rivers J.P.W. The nutritional role of indispensable amino acids and the metabolic basis for their requirement. Eur J Clin Nutr (1988), **42**, 367-393

Millward D.J, Jackson A.A, Price G.M. Human amino acids and protein requirements: current dilemas and uncertainties. Nutr Res Rev (1989), **2**, 109-132

Millward D.J. The metabolic basis of amino acid requirements. In: Protein-Energy Interactions Ed Scrimshaw N.S. & Schurch B. IDECG (1991)

Mitch WE, Lietman PS, Walser M. Effects of neomycin and kanamycin in chronic uraemic patients I urea metabolism. Kid Int (1977), **11**, 116-122

Mitch WE, Walser M. Effects of neomycin and kanamycin in chronic uraemic patients II nitrogen balance Kid Int (1977), **11**, 123-127

Miyoshi H, Okuda T, Fujita Y, Koishi H. Effect of dietary protein levels on urea utilisation in PNG highlanders. Jpn J Phys (1986), **36**, 761-771

Moran B.J. & Jackson A.A. Metabolism of 15N labelled urea in the functioning and defunctioned human colon. Clin Sci (1990a), **79**, 253-258

Moran B.J. & Jackson A.A. 15N-urea metabolism in the functioning human colon: luminal hydrolysis and mucosal permeability Gut (1990b), **31**, 454-457

Motil KJ, Matthews DE, Bier DM, Burke JF, Munro HN, Young VR. Whole body leucine and lysine metabolism: response to dietary protein intake in young men. Am J Phys (1981a), **240**, E712-E721

Motil KJ, Bier DM, Matthews DE, Burke JF, Young VR. Whole body leucine and lysine metabolism studie with [1-13C[leucine and [α-15N]lysine: response in healthy young men given excess energy intake. Metabolism (1981b), **30**, 783-791

Munro HN Carbohydrate and fat as factors in protein utilisation and metabolism. Phys Rev (1951), **31**, 449-488

Munro H.N. Historical introduction: the origin and growth of owr present concepts of protein metabolism. In: Mammalian Protein Metabolism Ed: Munro H.N. & Allison J.B. vol 1 (1964), Acad Press NY, p1-29

Muramatsu T, Kato M, Tasaki I, Okumaura J Enhanced whole body protein synthesis by methionine and arginine supplementation in protein starved chicks. Br. J. Nutr (1986), **55**, 635-641

Murdaugh H.V. Urea metabolism during low protein intake studies in dog and man. Exp Med Cong Ser (1970), **195**, p471-477

Oomen H.A.P.C. Interrelationship of hte human interatinal flora and protein utilisation Proc Nutr Soc (1970), **29**, 197-206

Pacy PJ, Price GM, Haaliday D, Quevedo MR, Millward DJ. Nitrogen homeostasis in man: the diurnal responses of protein synthesis and degradation and amino acid oxidation to diets with increasing protein intakes. Clin Sci (1994), **86**, 103-118

Parsons HG, Wood MM, Pencharz PB. Diurnal variaton in urine [15N[urea content, estimates of whole body protein turnover and isotope recycling in healthy meal fed children with cystic fibrosis Can J. Pharm Phys (1983), **61**,72-80

Passonneau JV, Lowry OH. The role of phosphofructokinase in metabolic regulation Adv Enz Reg (1964), **2**, 265-274

Persaud C, McDermott J, deBenoist B, Jackson AA. The excretion of 5-oxoproline in urine as an index of glycine status during normal pregnancy. Br J Obs Gynaec (1989), **96**, 440-444

Persaud C. PhD Thesis, University of Southampton, 1995

Phillips GB, Schwartz R, Gabudza GJ, Davidson CS. The syndrome of impending hepatic coma in patients with cirrhosis of the liver given certain nitrogenous substances.New Eng J. Med (1952), **247**, 239-246

Picou D. & Phillips M. Urea metabolism in malnourished and recovered children receiving a high or low protein diet. Am J Clin Nutr (1972), **25**, 1261-1266

Pogson CI. Adipose tissue pyruvate kinase properties and interconversion of two active forms. Biochem J (1968), **110**, 67-77

Price GM, Halliday D, Pacy PJ, Quevedo MR, Millward DJ Nitrogen homeostasis in man: influence of protein intake on the amplitude of diurnal cycling of body nitrogen Clin Sci (1994), **86**, 91-102

Quevedo MR, Price GM, Halliday D, Pacy PJ, Millward DJ. Nitrogen homeostasis in man: Diurnal changes in nitrogen excretion, leucine oxidation and whole body leucine kinetics during a reduction from a high to a moderate protein intake. Clin Sci (1994), **86**, 185-193

Rand W.M, Young V.R, Scrimshaw N.S. Change of urinary nitrogen excretion in response to low protein diets in adults. Am J Clin Nutr (1976), **29**, 639-644

Rennie MJ, Edwards RHT, Halliday D, Matthews DE, Wolman SL. Muscle protein synthesis measured by stable isotope techniques in man: the effects of feeding and fasting. Clin Sci (1982), **63**, 519-523

Reynolds MS, Steel DL, Jones EM, Bauman CA. Nitrogen balances of women in maintained on various levels of methionine and cystine. J. Nutr (1958), **64**, 99-111

Richards P. Utilisation of ammonia nitrogen for protein synthesis in man, and the effect of protein restriction and uraemia Lancet, 21 Oct 1967, 845-849

Richards P. Nutritional potential of nitrogen recycling in man Am J Clin Nutr (1972), **25**, p615-625

Rose W.C. The nutritive significance of the amino acids. Phys Rev (1938), **18**, p109-136

Rose W.C, Smith L.C, Womack M, Shane M. The utilisation of the nitrogen of ammonium salts, urea and certain other aompounds in the synthesis of non-essential amino acids in vivo. J Biol Chem (1949), **181**, 307-316

Rose W.C. & Wixom R.L. The amino acid requirements of man XVI the role of nitrogen intake J Biol Chem (1955), **217**, 997-1004

Rose W.C. & Dekker E.R. Urea as a source of nitrogen for the biosynthesis of amino acids J Biol Chem (1956), **223**, 107-121

Rose WC, Coon MJ, Lockhart HB, Lambert CF. The amino acid requirements of man XI the threonine and methionine requirements. J Biol Chem (1955), **215**, 101-110

Rose W.C. The amino acid requirements of adult man. Nutr Abs Rev (1957), **27**, 631-647

Rosman JB, Meijer S, Sluiter WJ, Terwee PM, Piers-Becht TPM, Donker AJM. Prospective randonised trial of early protein restriction in chronic renal failure. Lancet (1984).

Roth JS, Allison JB The effect of feeding excess glycine, l-arginine and dl-methionine to rats on casein diet. Proc Soc Exp Biol Med (1949), **70**, 327-330

Schimke R.T. Adaptive changes of urea cycle enzymes in the rat. J Biol Chem (1962), **237**, 459-468

Scrimshaw NS, Young VR, Schwartz R, Piche ML, Das JB. Minimum dietary essential amino acid-to-total nitrogen ratio for whole egg protein fed to young men J Nutr (1966), **89**, 9-18

Scrimshaw NS, Young VR, Huang PC, Thanangku O, Cholakos BV. Partial dietary replacement of milk protein by nonspecific nitrogen in young men J. Nutr (1969), **98**, 9-17

Scrimshaw NS, Hussein MA, Murray E, Rand WM, Young VR. Protein requirements of man: variations in obligatory urinary and faecal nitrogen losses in young men. J. Nutr (1972), **102**, 1595-1604

Scrimshaw N.S, Schurch B. Protein: energy interactions. IDECG (1991)

Sheng YB, Badger TM, Asplund JM, Wixom RL. Incorporation of $^{15}NH_4Cl$ into histidine in adult man. J Nutr (1977), **107**, 621-630

Shigesada K, Aoyagi K, Tatibana M. Role of acetylglutamate in ureotelism. Eur. J. Biochem (1978), **85**, 385-391

Shipley R.A. & Clarke R.E. Tracer methods for in vivo kinetics, NY Acad Press (1972), 145-162

Smith C.J, Bryant M.S, Bryant M.P. Introduction to metabolic activities of intestinal bacteria. Am J Clin Nutr (1979), **32**, 149-157

Snyderman S.E, Emmett-Holt L, Dancis J. 'Unessential' nitrogen: a limiting factor for human growth. J Nutr (1962), **78**, 57-72

Steffee W.P, Anderson C.F, Young V.R. An evalutation of the diurnal rhythm of urea excretion in healthy young adults. J Par Ent Nutr (1981), **5**, 378-384

Steinbrecher H. Urea kinetics in normal breast fed infants measured in their home. Proc Nut Soc (1994), **53**

Summerskill WHJ, Wolpert E. Ammonia metabolism in the gut. Am J Clin Nutr (1970), **23**, 633-639

Swendseid ME, Williams I, Dunn MS. Amino acid requirements of young women based on nitrogen balance data I the sulphur containing amino acids. J Nutr (1956), **58**, 495-505

Swendseid M.E, Harris C.L, Tuttle S.G. The effect of sources of non-essential nitrogen in young adults. J Nutr (1960), **71**, 105-108

Swendseid M.E, Watts J.H, Harris C.L. An evaluation of the FAO amino acid reference pattern in human nutrition I studies with young men. J Nutr (1961), **75**, 295-302

Swendseid M.E, Harris C.L, Tuttle S.G. An evaluation of the FAO amino acid reference pattern in human nutrition II studies with young women. J Nutr (1962), **77**, 391-396

Tanaka N et al. A pilot study for protein metabolism in the PNG highlanders. J Nutr Sci Vitaminol (1980), **26**, 247-259

Torrallardona D, Harris CI, Milne E, Fuller MF. Contribution of intestinal microflora to lysine requriements in non-ruminants. Proc Nut Soc (1993a), **52**, 153A

Torrallardona D, Harris CI, Milne E, Ronaasen V, Coates ME, Fuller MF. Contribution of intestinal microflora to lysine requirements in rats. Proc Nut Soc (1993b), **52**, 368A

Tripathy K, Klair S, Lotero H. Utilisation of exogenous urea nitrogen in malnourished adults. Metabolism (1970), **19**, 253-261

Uauy R, Scrimshaw N.S, Rand W.M, Young V.R. Human protein requirements: obligatory urinary and faecal nitrogen lossess and the factorial estimation of protein needs in elderly males. J Nutr (1978), **108**, 97-103

Ueda Y et al. An epidemiological study on strokes. Jpn J Publ Health (1979), **26**, 271

Varcoe R, Halliday D, Carson E.R, Richards P, Tavill A.S. Efficiency of utilisation of urea nitrogen for albumin synthesis by chronically uraemic and normal man. Clin Sci Mole Med (1975), **48**, 379-390

Visek WJ. Effects of urea hydrolysis on cell life span and metabolism. Fed Proc (1972), **31**, 1178-1193

Visek WJ. Some biological considerations in utilisation of non-specific nitrogen J Agric Fd Chem (1974), **22**, 174-184

Visek W.J. An update on concepts of essential amino acids. Ann Rev Nutr (1984), **4**, 137-155

Walser M, George J, Bodenlos LJ Altered proportions of isotopes of molecular nitrogen as evidence for a monomolecular reaction J. Chem. Phys (1954), **22**, 1146

Walser M. & Bodenlos L.J. Urea metabolism in man J Clin Invest (1959), **38**, 1617-1626

Walser M. Use of isotopic urea to study the distribution and degradation of urea in man. Exp Med Cong Ser (1970), 421-429

Walser M. Urea metabolism in chronic renal failure. J Clin Invest (1974), **53**, 1385-1392

Walser M. Urea metabolism. In: Nitrogen metabolism in man. Eds: Waterlow JC & Stephens JML. App Sci Pub (1981), p229-246

Walter JH, Leonard JV. Inborn errors of the urea cycle. Br J. Hosp Med (1987), 176-183

Waterlow JC. Observations on the mechanism of adaptation to low protein intakes Lancet, 23 Nov 1968, 1091-1097

Waterlow JC, Jackson AA. Nutrition and protein turnover in man. Br Med Bull (1981), **31**, 5-10

Waterlow JC. Protein turnover with special reference to man Quat J Exp Phys (1984), **69**, p409-438

Waterlow J.C. What do we mean by adaptation? In: Nutritional adaptation in man. Ed: Sir K Blaxter & Waterlow JC, John Libby Publishers, (1985), 1-11

Watford M. The urea cycle a two compartment system. Essays in Biochem (1991), **26**, 49-58

Watts J.H, Tolbert B, Ruff W.L. Nitrogen balances for young adult males fed two sources of non-essential nitrogen at two levels of total nitrogen intake Metabolism (1964), **13**, 172-180

Weller L.A, Calloway D.H, Margen S. Nitrogen balance of men fed amino acid mixtures based on Rose's requirements, egg white protein and serum free amino acid pattern J Nutr (1971), **101**, 1499-1508

Wheeler R.A, Jackson A.A, Griffiths D.M. Urea production and recycling in neonates J Paed Surg (1991), **26**, p575-577

Widdowson EM, Southgate DAT, Hey EN. Body composition of the feotus and infant. In: Nutrition and metabolism of the feotus and infant. Ed Visser HKA. The Hague. p169-177

Wolfe R.R. Measurement of urea kinetics in vivo by means of a constant tracer infusion of di-15N urea. Am J Phys (1981), **240**, pE428-E434

Wolfe RR. Radioactive and stable isotope tracers in biomedicine: principles and practice of kinetic analysis. Wiley Liss NY (1992), 119-144

Wolpert E, Phillips S.F, Summerskill W.H.J Transport of urea and ammonia production in the human colon Lancet, 25 Dec 1971, 1387-1390

Wretland KAJ, Rose WC. Methionine requirement for growth and utilisation of its optical isomers. J Biol Chem (1950), **187**, 697-703

Wright JB, Martin PG, Skellenger ML, Moschette DS Metabolic patterns in preadolescent children III sulphur balance on three levels of nitrogen intake. J Nutr (1960), **72**, 314-316

Wrong OM, Metcalfe-Gibson A, Morrison BI, Ng ST, Howard AV In vivo dialysis of faeces as a method of stool analysis I technique and results in normal subjects. Clin Sci (1965), **28**, 357-375

Wrong O, Houghton B.J, Richards P, Wilson D.R. The fate of intestinal urea in normal subjects and patients with uraemia. - Exp Med Cong Ser (1970), 461-469

Wrong OM, Edmunds CJ, Chadwick VS
The Large Intestine; Its role in homeostasis (1981)
MTP Press, Lancaster, 133-155

Wurtman R.J. Diurnal rythmns in mammalian protein metabolism In: Mammalian Protein Metabolism Ed Munro H.N.
Volume IV, Acad Press NY (1970)

Yang RD, Matthews DE, Bier DM, Wen ZM, Young VR. Response to alanine metabolism in humans to manipulations of dietary protein and energy intakes. Am J Clin Nutr (1986), **250**, E39-E46

Yeboah N, Ah-sing E, Badaloo A, Forrester T, JAckson A, Millward DJ. Transfer of ^{15}N from urea to the circulating lysine pool in the human infant. Proc Nut Soc (1995) In press.

Yokogoshi H, Yoshida A. Effect of supplementation of methionine and threonine to a protein free diet on urinary excretion of nitroge and tissue free amino acids in rats.
J. Nutr (1977), **107**, 783-791

You G, Smith CP, Kanai Y, Lee WS, Stelzner M. Cloning and characterisation of the vasopressin regulated urea transporter. Nature (1993), **365**, 844-847

Young VR, Scrimshaw NS. Endogenous nitrogen metabolism and plasma free amino acids in young adults given a protein free diet. Br J Nutr (1968), **22**, 9-20

Young V.R, Taylor V.S.M, Rand W.M, Scrimshaw N.S. Protein requirements of man: efficiency of egg protein utilisation at maintenance and sub-maintenance levels in young men. J Nutr (1973), **103**, 1164-1174

Young VR, Robert JJ, Motil KJ, Matthews DE, Bier DM. Protein & Energy intake in relation to protein turnover in man. In: NItrogen metabolism in man. Eds Waterlow JC & Stephens JML. App Sci Pub (1981) p419-435

Young VR, Puig M, Queiroz E, Scrimshaw NS. Evaluation of the protein quality of an isolated soy protein in young men: relative nitrogen requriements and effect of methionine supplementation. Am J Clin Nutr (1984), **39**, 16-24

Young VR, Moldawer LL, Hoerr R, Bier DM. Mechanisms of adaptation to protein malnutrition. in: Nutritional adaptation in man Ed Sir K Blaxter, Waterlow JC. John Wiley Pubs (1985), 189-217

Young V.R. Nutritional balance studies: indicators of human requirements or of adaptive mechanisms? J Nutr (1986), **116**, 700-703

Young V.R. & Bier D.M. Amino acid requirements in the adult human: how well do we know them? J Nutr (1987), **117**, 1484-1487

Young V.R. & Bier D.M.A. Kinetic approach to the determination of human amino acid requirements
Nut Rev (1987), **45**, p289-298

Young V.R, Bier D.M, Pellet P.L. A theoretical basis for increasing current estimates of the amino acid requirements in adult man Am J Clin Nutr (1989), **50**, p80-92

Young VR, MArchini JS. Mechanisms and nutritional significance of metabolic responses to altered intakes of protein and amino acids with reference to nutritional adaptation in man Am J Clin Nutr (1990), **51**, 270-289

Young VR, Pellet PL. Current concepts concerning indispensable amino acid needs in adults and their implications for international nutrition planning. Food & Nutr Bull (1990), **12**, 289-300

Young VR, Wagner DA, Burnini R, Storch KJ. Methionine kinetics and balance at the 1985 FAO/WHO/UNU intake requirements in adult men studies with L-[$2H_3$-methyl-1 13C] methionine as a tracer. Am J Clin Nutr (1991), **54**, 377-385.

Yu YM, Yang RD, Matthews DE, Wem ZM, Burke JF, Bier DM, Young VR. Quantitative aspects of glycine and alanine nitrogen metabolism in postabsorptive young men: effects of level of nitrogen and dispensable amino acid intake J Nutr (1985), **115**, 399-410

Zezulka AY, Calloway DH. Nitrogen retention in men fed varying levels of amino acids from soy protein with or without added l-methionine J. Nutr (1976a), **106**, 212-221

Zezulka AY, Calloway DH. Nitrogen retention in men fed isolated soybean protein supplemented with l-methionine, d-methionine, n-acetyl-l-methionine or inorganic sulphate J Nutr (1976b), **106**, 1286-1291

Dietary reference values for food and energy and nutrients for the United Kingdom.
Report on health and social subjects 41, HMSO (1991)

Energy and Protein requirements
Report of FAO/WHO/UNU expert consultation
WHO technical report series 724, Geneva (1985)