UNIVERSITY OF SOUTHAMPTON

THE MODULATORY EFFECTS OF DIETARY SULPHUR AMINO ACIDS, TRYPTOPHAN AND ARGININE IN YOUNG GROWING RATS UNDERGOING AN INFLAMMATORY RESPONSE

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ABSTRACT

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THE MODULATORY EFFECTS OF DIETARY SULPHUR AMINO ACIDS, TRYPTOPHAN AND ARGININE IN YOUNG GROWING RATS UNDERGOING AN INFLAMMATORY RESPONSE

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Following infection or other inflammatory stimuli, an acute phase response occurs in which cytokines and oxidant molecules are produced to help in the removal of causative agent and repair any damage caused by the insult. However, inappropriate production of cytokines and oxidant molecules could carry the risk of damaging or destroying the host. The presence of adequate amounts of antioxidant defences, such as glutathione, help the body in scavenging oxidant molecules and regulating cytokines production. Since the synthesis of many compounds containing sulphur amino acids (cysteine and methionine), such as glutathione and wide range of acute phase proteins, are increased after exposure to an inflammatory stimulus, the availability of sulphur amino acids may have a strong influence on the ability of the host to successfully resist invading organisms.

However, Reeds *et al.* (1994) have calculated that the profile of amino acids released from skeletal muscle during the acute phase response is relatively deficient in the aromatic amino acids (phenylalanine, tryptophan and tyrosine) in relation to the amino acid composition of the acute phase proteins. Furthermore, it was hypothesised that the imbalance between the proportions of amino acids released from muscle and the proportions needed for acute phase protein synthesis, was responsible for the negative nitrogen balance during the inflammatory response.

Arginine supplementation has also been shown to have immunoenhancing effects in normal subjects, and in clinically ill patients. In rats, addition of arginine reduced the extent of the N negative nitrogen balance following bone fracture.

The extent to which dietary intervention, by correcting deficiencies in sulphur amino acids, tryptophan, and arginine intake, improves antioxidant defences and modulates the metabolic response to infection was examined in this thesis using a young rat model. Rats were given an inflammatory challenge by intraperitoneal injection of endotoxin, and were compared with *ad libitum* and pair-fed controls.

Glutathione (GSH) concentrations in various organs (liver, lung, spleen and thymus), and hepatic protein content decreased in animals fed the low-protein diets. In endotoxin-treated rats, the magnitude of the increase of the acute phase protein, α-1 acid glycoprotein, was reduced in animals fed the low-protein diets.

Addition of the sulphur amino acid, methionine, to the low-protein diets, restored GSH concentrations and increased hepatic protein content in endotoxin-treated animals and in animals fed *ad libitum*. In endotoxin-treated rats, addition of methionine to the low-protein diets reduced (not statistically significant) the number of neutrophils in lung, while in *ad libitum* and in pair-fed animals, addition of methionine increased (not statistically significant) the number of neutrophils. Furthermore, before starting treatments, addition of methionine to the low-protein diets improved growth.

In general, there was no indication that addition of tryptophan or arginine had marked effects on the acute inflammatory response after exposure to the endotoxin.

To conclude, dietary methionine is the most important amino acid regarding the ability to modulate the inflammatory response.

CONTENTS

LIST OF CONTENT

CHAPTER ONE

CHAPTER TWO

CHAPTER THREE

CHAPTER FOUR

CHAPTER FIVE

CHAPTER SIX

CHAPTER SEVEN

CHAPTER EIGHT

LIST OF TABLES

CHAPTER ONE

CHAPTER THREE

CHAPTER FOUR

CHAPTER FIVE

CHAPTER SIX

CHAPTER SEVEN

CHAPTER EIGHT

APPENDIX A

LIST OF FIGURES

CHAPTER ONE

CHAPTER TWO

CHAPTER THREE

CHAPTER FOUR

CHAPTER FIVE

CHAPTER SIX

CHAPTER SEVEN

CHAPTER EIGHT

PREFACE

1) Alhamdan, A., Grimble, R.F. (1997): The effect of dietary cysteine adequacy on glutathione metabolism during inflammation in rats (Abstract-page R490). 16th International Congress of Nutrition-Montreal-Canada.

2) Alhamdan, A., Grimble, R.F. (1998): The modulatory effect of dietary sulphur amino acids in young growing rats undergoing an inflammatory response (oral presentation). Mini-Symposium on Sulphur: The Globale Cycle, Metabolism and Nutrirtion. Sponsered by The Rank Prize Funds. Held at The Wordsworth Hotel, Grasemere, Cumbria. 14th to 17th July, 1998.

3) Alhamdan, A., Grimble, R.F. (1999): The modulatory effect of dietary sulphur amino acids and tryptophan in young growing rats undergoing an inflammatory response (oral presentation). Geoffrey Taylor-Postgraduate Symposium: School of Biological Sciences (Third Year Symposium 1999).

4) Alhamdan, A., Grimble, R.F. (1999): Glutathione metabolism in endotoxin-treated rats fed low-protein diets with and without sulphur amino acid supplementation (oral presentation). Functional Foods '99-claims and evidence. British Nutrition Foundation. Page 36 in the abstracts Booklet. Held at Withersdane Conference Centre, Wye College, University of London, Kent, UK. 14th-15th April 1999.

5) Alhamdan, A., Grimble, R.F. (2000): The effect of dietary methionine supplementation on hepatic glutathione metabolism, hepatic protein content, and α-1 acid glycoprotein in endotoxin-treated rats fed low-protein diets. Proceedings of the Nutrition Society 59:45A.

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LIST OF ABBREVIATIONS

°C	Centigrade
µmol	micromole
APR	Acute phase response
BCA	Bicinchoninic acid
B-HM	Beatin-homocysteine methyltransferase
C-βS	Cystathionine β-synthase
CRF	Corticotrophin releasing factor
CRHs	Counter regulatory hormones
CRP	C-reactive protein
D.water	Distilled water
ETC	Electron transport chain
g	Gram
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced form of glutathione
GSSG	Oxidised form of glutathione
H_2O_2	Hydrogen peroxide
HDL	High density lipoprotein
HOCl	Hypochlorous acid
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
IL1-ra	Interleukin 1 receptor antagonist
KD	Kilodalton
KDO	Ketodeoxy octanoic acid
kg	Kilogram
LPS	Lipopolysaccharide
mg	milligram
ml	millilitre
N	Nitrogen
NAC	N-acetyl-L-cysteine
NH_3	Ammonia
nmol	nanomole
$NO^{\bullet}$	Nitric oxide
$O_2^{\bullet-}$	Superoxide
$OH^{\bullet}$	Hydroxyl radical
OTC	L-2-oxothiazalidine-4-carboxylate
PEG	Polyethylene glycol
PMN	polymorphonucleocytes
PUF	Polyunsaturated fatty acid
ROS	Reactive oxygen species
S-AM	S-adenosyl methionine
SE	Standard error of the mean
SH	Thiol group
S-MTHM	S-methyl tetrahydrofolate homocysteine methyltransferase
SO_4	Inorganic sulphate
TNF	Tumor necrosis factor
TNF-a	Tumor necrosis factor antagonist

CHAPTER ONE

1. Literature Review

1.1 Introduction

Inflammation is a complex process and a morbid condition of the body initiated by tissue damage caused by various of factors. Inflammation may be caused by mechanical injury (e.g. surgery), physical injury (e.g. burn), chemical injury (e.g. exposure to a corrosive chemical), immunological injury (e.g. hypersensitivity reactions) and biological injury (e.g. infection) (Benjamini & Leskowitz, 1991). Inflammation is closely related to serious health problems, for example following trauma which is the principle cause of death between the ages of 1 and 40. In Great Britain injured patients occupy more hospitals beds than patients with cancer and heart disease combined (Westaby, 1989). Infection is still the major cause of death in developing countries. Death due to infectious diseases reaches about 30 to 50% of the population in some developing countries (Playfair, 1995).

Cachexia, weight loss and wasting of peripheral tissues are the main characteristics of diseases associated with an inflammatory stimulus (Fong *et al.* 1989; Tracey, 1992). The difference between cachexia associated with an inflammatory stimulus (e.g. infection) and weight loss caused by starvation is that in cachexia there is a wasting of peripheral proteins and a conservation of liver proteins, while in starvation there is a conservation of skeletal muscle proteins and a loss of liver proteins (Brennan & Ekman, 1984; Wilmore, 1991; Tracey, 1992). Fong *et al.* (1989) have demonstrated that when rats were injected with inflammatory agents (Lipopolysaccharide, Tumour necrosis factor, or Interleukin-1) there was wasting of muscular proteins and preservation of liver proteins. When these rats were compared with a pair-fed controls (rats given the same amount of food eaten by animals receiving inflammatory stimuli), the pair-fed animals showed a relative preservation of skeletal muscle protein and a loss of liver proteins.

Following an inflammatory stimulus (e.g. bacteria, injury), cytokines and oxidant molecules are produced to aid in the removal of causative agent and repair

any damage caused by the inflammatory insult (Roitt *et al.* 1996). Cytokines and oxidant molecules have the ability to enhance each other production. Cytokines enhance free radical production from macrophages (Lamas *et al.* 1991), and free radicals enhance cytokine production via activation of the nuclear transcription factor NFκB (Schreck *et al.* 1991). NFκB is a protein, which has been found in the cytoplasm of various cell types, such as B and T lymphocyte, macrophages, and monocytes (Baeurele & Baltimore, 1991). NFκB has the ability to up-regulate cytokine production by promoting the transcription of mRNA for cytokines within cytokine producing cells (Peristeris *et al.* 1992; Pfizenmaier *et al.* 1992; Schreck *et al.* 1991). NFκB is present in the cytoplasm of unstimulated cells in a complex with its inhibitor, IκB (Baeuerle & Baltimore, 1988). When the cell is stimulated, for example with oxidant molecules or with cytokines, NFκB released from its inhibitor, IκB, migrates to the nucleus, and activates its target genes (Ghosh & Baltimore, 1990).

Although, production of oxidant molecules and cytokines after infection could be beneficial, inappropriate production of cytokines and oxidant molecules could carry the risk of destroying the host (Tracey & Cerami, 1993; Halliwell *et al.* 1988). Therefore, the presence of adequate antioxidant defences within the host could play a role in protecting the body from potentially toxic substances such as oxidant molecules, for example, by converting them to non-harmful substances, e.g. water, and by reducing the ability of oxidant molecules to enhance the activity of the NFκB, thereby helping to regulate cytokine production.

A major question asked by this thesis is the extent to which dietary intervention, either by changing dietary protein level, or by adding specific amino acids, can improve antioxidant defences, and modulate the metabolic response to inflammation.

Glutathione and sulphur amino acids will be discussed in detail in this review, since a number of studies suggest an increased requirement for sulphur amino acids and glutathione during an inflammatory response. In addition, a description of

cytokines, oxidant molecules, acute phase proteins, and their effects will also be discussed.

1.2 Cytokines

Cytokines are polypeptides with low molecular weight, rarely more than 15-30 Kilodalton (KD) in size. Cytokines comprise interleukins 1-17 (IL1-17), Tumour necrosis factor (TNF), and interferons (IFN). They were originally thought to be produced by lymphoid cells only, and for this reason, they were named lymphokines. It is well established that other cells are also capable of producing these "kines", and they acquired the name cytokines (Dinarello & Mier, 1987). Activated phagocytic immune cells, T and B-lymphocytes, fibroblasts, and various endothelial cells are all capable of synthesising cytokines (Dinarello & Mier, 1987; Dinarello, 1986; Miossec & Ziff, 1986). A number of cytokines and their main features are summarised in Table 1.1 (Roitt *et al.* 1996). As can be seen from the table, they exert a wide range of biological effects.

Cytokines can stimulate the production of each other. Any change in the production of one cytokine can have a profound effect on the production of other cytokines (Meydani, 1990).

Cytokines act as hormone-like molecules within the immune system. In the computer world, for example, an instrument called a modem is used to connect one computer to other. Similarly in the immune system, cytokines act as a 'biological modem', which allows the immune cells to be in permanent communication with each other.

One of the beneficial functions that the body can obtain from producing cytokines, in response to an inflammatory stimulus, is to destroy invading pathological organisms and restore damaged tissue (Grimble, 1994a; Grimble, 1994b). This is also one of the functions of oxidant molecules. However, excessive or inappropriate production of cytokines frequently leads to mortality and morbidity (Tracey & Cerami, 1993).

Table 1.1 Cytokines and their main features *

Cytokine	Molecular weight	cell source (s)	main cell target (s)	main actions
IFN-γ	40-50 000 (dimer)	T Cells, NK cells	lymphocytes, monocytes,tissue cells	immunoregulation, B cell differentiation, some antiviral action
IL-1-α, IL-1β	33 000 (precursor) 17 500 (mature)	monocytes, dendritic cells, some B cells, fibroblasts, epithelial cells, endothelium, astrocytes, macrophages	thymocytes, neutrophils, T and B cells, tissue cells	immunoregulation, inflammation, fever
IL-2	15 000	T cells, NK cells	T cells, B cells, monocytes	proliferation, activation
IL-3	150 000	T cells	stem cells, progenitors	pan-specific colony stimulating factor
IL-4	15 000	T cells	B cells, T cells	division and defferentiation
IL-5	20 000	T cells	B cells, eosinophils	differentiation
IL-6	20 000	macrophages, T cells, fibroblasts, some B cells	T cells, B cells, thymocytes, hepatocytes	differentiation, acute phase protein synthesis
IL-8 (family)	8000	macrophages, skin cells	granulocytes, T cells	chemotaxis
TNF-α TNF-β (lymphotoxin)	50 000 (trimer)	macrophages, lymphocytes	fibroblasts, endothelium	inflammation, catabolism (cachexia), fibrosis; production of other cytokines (IL-1, IL-6, GM-CSF)

*** source (Roitt *et al.* 1996).**

1.2.1 The role of cytokines during the immune response

At the onset of infection, cytokine producing cells, e.g. macrophages, are activated either by phagocytosis of the invading microorganism or by exposure to its products or toxins. Both of which resulted in the synthesis and release of cytokines (Dinarello, 1984).

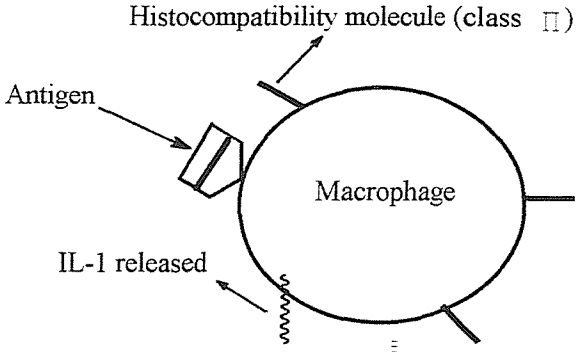
When antigens are displayed on the surface of macrophages, or other antigen presenting cells, in a physical association with a class ∏ histocompatibility molecule (DR), T-cells can recognise antigens. The receptors of T-cells interact with the antigen–DR complex and become activated. Macrophages secrete IL-1 which stimulates T-cells to grow, become activated, and to secrete a variety of cytokines,

such as IL-1, 2, 3, and 4, B cell differentiating factor, and interferon-γ (IFN-γ). IL-1 increases the synthesis of IL-2,3, and 4, γ-interferon, and IL-2 receptors. Clonal expansion of activated T-cell is influenced by IL-2. IL-3 stimulates the marrow stem cells to produce erythrocytes (hematopoiesis). IL-4, B-cell differentiating factor, IL-1, IL-2, and γ-interferon provide signals necessary for B-cells maturation. Finally, γ-interferon, released from activated T-cell, increases the recognition of T-cell to the antigen by enhancing the HLA-DR expression on the macrophages (see Figure 1-1).

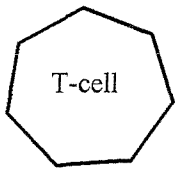
Cytokines like IFN-α, IFN-γ, IL-2, and IL-4 stimulate and modify immune function following an inflammatory stimulus. Other cytokines like IL1, IL-6, TNF-α, and TNF-β not only stimulate and modify immune functions, but also initiate metabolic changes in the macro and micro nutrients following infection, cancer, surgery, and injury (Charters & Grimble, 1989; Cousins & Leinart, 1988; Dinarello, 1984; Feingold *et al.* 1989), see Figure 1-2.

Figure 1-1 T-cell activation & cytokine production

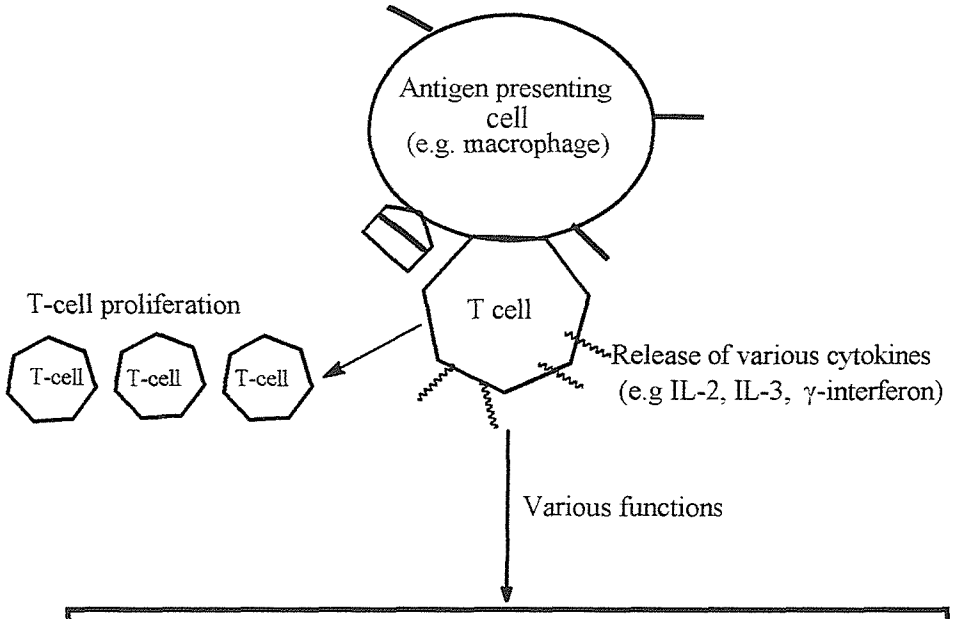
1)Activation of macrophage by antigen.



2) Recognition of T-cell to macrophage



3) Antigen presenting cell bound to T-cell

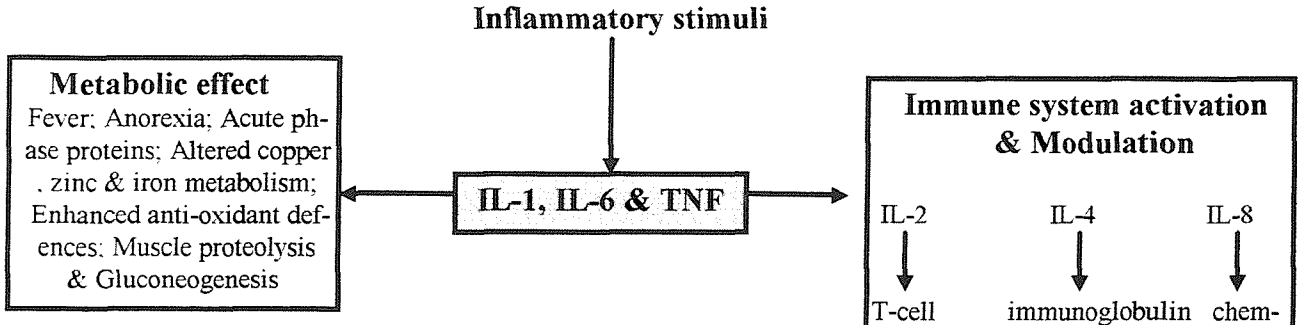


1) Stimulates B-lymphocyte to produce antibody.
2) B-cell activation and proliferation.
3) Macrophage activation.
4) T-8 cytotoxic cell produces substances to destroy the antigen-bearing cell.
5) Cytokines produced help in:
   a) Enhancing the recognition of T-cell to antigen.
   b) B-cell maturation.
   c) Clonal expansion of T-cell.

The metabolic effect of IL-1, IL-6, and TNF can be direct via interaction with tissues, and indirect via stimulation of the endocrine and central nervous system (Beutler & Cerami, 1988; Grimble, 1990).

As IL-1, IL-6, and TNF are the cytokines primarily responsible for the changes in nutrient metabolism, the properties of these cytokines will be discussed further.

Figure 1-2 Influences of pro-inflammatory cytokines upon the immune system and on metabolism following inflammatory stimuli *



*** Adapted from (Grimble:1994b).**

1.2.2 Interleukin 1 (IL-1)

Interleukin 1 was previously known as endogenous pyrogen, lymphocyte activating factor and catabolin (Roitt *et al.* 1996). It can be produced by many cells, such as endothelial cells, T and B cells (Golub & Green, 1991), but most abundantly by macrophages (Roitt *et al.* 1996). T and B cell activation is enhanced by IL-1 (Golub & Green, 1991). IL-1 stimulates T-cell to grow, and initiates the secretion of a variety of cytokines (Nossal, 1987). IL-1 activates T-cells, by enhancing the

recognition of T-cells, by antigen presenting cells (Dinarello & Mier, 1987). IL-1 also stimulates the production of prostaglandins and degradative enzymes (e.g. collagenase), and this function is believed to be responsible for the destruction of cartilage and bone following infection and in inflammatory diseases (Roitt *et al.* 1996). Fever-induced anorexia is also believed to be due to the action of IL-1 (Dinarello & Wolff, 1982; Roitt *et al.* 1996). The rise in body temperature might help the host in fighting infectious diseases, since many viruses and bacteria grow and replicate well at normal body temperature. Therefore, resetting the temperature of the body to be higher than normal (37 °C) provides an unsuitable environment for pathogen replication. Furthermore, antibody and interferon production, and phagocytosis, are enhanced by increasing body temperature. In the liver, IL-1 stimulates the production of acute phase proteins (Dinarello, 1984; Roitt *et al.* 1996). IL-1, in addition to other cytokines, is also able to stimulate the production of counter regulatory hormones, glucagon, cortisol, adrenaline, and growth hormone, indirectly, by enhancing the production of releasing factors such as corticotrophin releasing factor (CRF) (reviewed in Grimble, 1990).

1.2.3 Interleukin 6 (IL-6)

Interleukin 6 was previously known as B-cell differentiating factor or hepatocyte stimulating factor. It can be produced by macrophages, T cells, B cells, and fibroblast (Roitt *et al.* 1996). The main biological actions of IL-6 are stimulating B cell differentiation, and increasing immunoglobulin synthesis and secretion (Sehgal *et al.* 1987). It promotes growth of hybridomas, supports growth of T-cells and thymocytes, stimulates the production of acute-phase proteins in the liver (Heinrich *et al.* 1990), and enables B cells to differentiate into antibody-forming cells (Roitt *et al.* 1996).

1.2.4 Tumour necrosis factor (TNF)

Tumor necrosis factor, or cachectin, is a 17 KD protein produced from macrophages, T cells, B cells, natural killer cells, mast cells, eosinophils, astrocytes, and Kupffer cells (Tracey, 1994). The demonstration that TNF is one of the

endogenous mediators of the host response to inflammation has only occurred since 1980. Thereafter, large amounts of data, both in vivo and in vitro studies, have shown that most of the catabolic changes, which occur after the exposure to an inflammatory stimulus, can be reproduced by TNF (Moldawer *et al.* 1988). The biological effects of TNF on various organs are shown in Table 1.2.

Table 1.2 The biological effects of TNF on various organs *

Tissue or cell type	Biological response
Brain	1- Anorexia. 2- Increases adrenocorticotropin, growth hormone, prolactin. 3- Decreases thyroid stimulating hormone.
Muscle	1- Net whole body protein catabolism. 2- Increases efflux of amino acids from skeletal muscle. 3- Reduction of resting transmembrane potential. 4- Suppression of glucose transferase-4. 5- Glycogenolysis. 6- Lactate efflux.
Adipose	1- Suppression of lipoprotein lipase. 2- Increased free fatty acid efflux. 3- Increased lipolysis. 4- Decreased lipogenesis.
Liver	1- Acute-phase protein synthesis. 2- Decreased albumin synthesis. 3- Increased lipogenesis. 4- Enhanced glucagon-mediated amino acid transport. 5- Increased gluconeogenesis.

***Source: (Tracey, 1994).**

1.2.5 Cytokine receptors

Cytokines can induce their metabolic effects in cells via high affinity membrane receptors. When less than 10% of the total membrane receptor number is occupied by cytokines, the nucleus can receive adequate signals from cytokines, and maximum cellular biological responses occur (Tracey & Cerami, 1993). Due to these high affinity cytokine receptors, cytokines are able to induce their metabolic effects even at low concentrations (Roitt *et al.* 1996).

1.2.6 Innate control systems for preventing overexpression of cytokines

In response to an inflammatory stimulus, cytokines are produced with the purpose of providing nutrients for the immune system and increasing the resistance of the host against invading organisms. However, overproduction of cytokines leads to exhaustion of host nutrients and exerts damage to the tissue, as mentioned earlier. Excessive or overproduction of cytokines has been associated with morbidity and mortality in a wide range of diseases, such as malaria, asthma, cancer, meningitis, inflammatory bowel disease, rheumatoid arthritis (Kwaitkowski *et al.* 1990; Broide *et al.* 1992; Balkwill *et al.* 1987; Waage *et al.* 1989). Furthermore, excessive production of cytokines has been shown to have a role in the pathogenesis of multiple sclerosis, atherosclerosis, and Alzheimer's disease (Chofflon *et al.* 1992; Hajjar & Pomerantz, 1992; Bauer *et al.* 1992). Therefore, to obtain maximum beneficial effects from cytokines with minimum tissue damage, the amount of cytokines produced should be well controlled. The question here is: Do we have mechanisms which can control excessive production of cytokines? The answer is yes; When cytokines are produced, they bring about various metabolic changes, which in turn initiate mechanisms that downregulate and prevent over or unnecessary expression of cytokines.

IL-1 and TNF enhance the production of two types of cytokine inhibitory proteins namely, the receptor antagonist for IL-1(IL1-ra), and the cytokine antagonist for TNF (TNF-a). IL1-ra, structurally related to IL-1, is a protein produced by immunoglobulin G (IgG), adherent monocytes and keratinocytes. IL1-

ra binds to IL1 receptors, thereby reducing the number of receptors available for IL-1 to bind with. TNF-a is a soluble serum fragment generated by enzymatic cleavage from the extra cellular domain of the cytokine receptor. This shed receptor domain binds to TNF, thereby blocking its access to target receptors in the membrane. Consequently, as a result of the actions of these two cytokine inhibitory proteins, the metabolic effects produced by IL-1 and TNF to the host cells will be reduced (review, Roitt *et al.* 1996; Burger & Dayer, 1995).

A number of antioxidant compounds and enzymes are increased following TNF and IL-1 production. Consequently, the oxidant molecules ability to enhance the activity of the NFκB will be reduced, thereby down-regulating cytokine production. When young rats were injected with an inflammatory stimulus (TNF) glutathione (GSH) concentration in the liver was increased significantly (Hunter & Grimble, 1994). Wong & Goeddel (1988) have reported that treatment with TNF-α , TNF-β, IL-1α and IL-β were observed to increase the production of manganous superoxide dismutase. Superoxide dismutase protects the cells from the toxicity of superoxide radicals both in vivo and in vitro (McCord & Fridovich, 1969). Ceruloplasmin, which functions as an antioxidant defence (Goldstein *et al. 1*979), has also been reported to be increased by IL-1 in rats (Barber & Cousins, 1988).

Glucocorticoid production, due to the effect of cytokines on CRF, down-regulates the production of cytokines (Tracey & Cerami, 1993). Beutler *et al.* (1986) have shown that when macrophages, isolated from BALB/c mice, were treated with dexamethasone (a glucocorticoid hormone), there was suppression of cachectin (TNF) production, if the hormone was introduced prior to or at the same time as endotoxin.

Other cytokines, like IL-4 and IL-10, were also have been shown to down-regulate IL-1 and TNF production (Burger & Dayer, 1995).

1.2.7 Bacterial endotoxin (lipopolysaccharide)-a potent stimulator of cytokine production

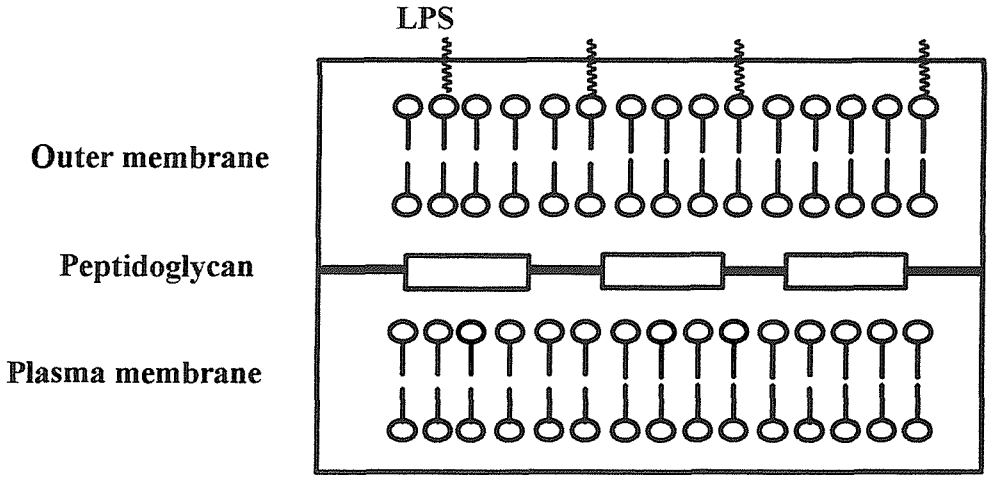
Bacterial endotoxin {lipopolysaccharide (LPS)} is the most widely studied agent inducing cytokine production (Tracey & Cerami, 1993). LPS is an integral part of the gram-negative bacteria. Gram negative bacteria consist of three layers (Figure 1-3): an outer membrane, a thin layer of peptidoglycan, and the plasma membrane. LPS located in the outer membrane, and accounts for approximately 40% of cell surface area. LPS is made up of three main parts (reviewed in Bannister *et al.* 1996) (Figure 1-4):

1) The core region, lipid A, which is responsible for the main toxic events and acts by stimulating macrophages to produce cytokines.

2) An oligosaccharide region linked to lipid A via the sugar compound, ketodeoxy octanoic acid (KDO).

3) A long polysaccharide chain, responsible for the antigenic specificity for the LPS, attached to the oligosaccharide region.

Figure 1-3 Structure of gram negative bacteria

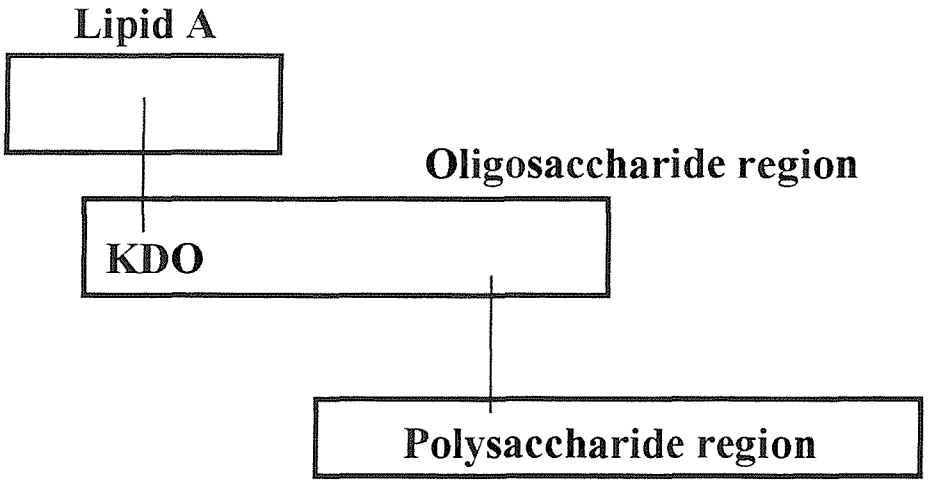


LPS is transported in the blood as a complex with the lipid transport compound, high-density lipoprotein (HDL). Munford *et al.* (1981) reported that

organs which normally take-up HDL, e.g. liver, are those organs with high risk of exposure to high concentration of endotoxin.

The production of TNF, by macrophages and monocytes, can be initiated by endotoxin (Beutler *et al.* 1986; Sariban *et al.* 1988). Endotoxin was also found to stimulate IL-1 production from macrophages, monocytes, neutrophils and endothelial cells (Miossec & Ziff, 1986; Tiku *et al.* 1986). TNF and IL-1 are also found in the serum of patient with sepsis, in whom the likely cause was LPS (Giradin *et al.* 1988).

Figure 1-4 The main structural parts of lipopolysaccharide



1.2.8 Cytokines and their metabolic effect on protein metabolism

Infection is associated with an increase in muscle protein breakdown and negative nitrogen balance (Beisel, 1975). The amino acids released from muscle are used by visceral organs (e.g. liver) to provide carbon skeleton for gluconeogenesis

(Powanda, 1977), and in the synthesis of acute phase proteins (Powanda, 1977; Hunter & Grimble, 1994).

Following infection or injury, IL-1, IL-6, IL-8 and TNF initiate wasting of peripheral tissues resulting from the loss of tissue protein, lipid and micro-nutrients (Grimble, 1994b). The most studied cytokines involved in altering protein metabolism are IL-1, IL-6, and TNF (Charters & Grimble, 1989; Dinarello, 1984; Hack *et al.* 1996). Increased production of cytokines, particularly, IL-1, IL-6, and TNF-α leads to a significant alteration in muscle protein metabolism. The alteration in protein metabolism could be due to both enhanced proteolysis and decreased protein synthesis (Baracos *et al.*1983; Charters & Grimble, 1989). After exposure to an inflammatory stimulus, an increase in muscle protein catabolism occurred, and the amino acids released are used for glucose, glutathione and acute phase protein synthesis (Brenner *et al.* 1990; Fong *et al.* 1989; Fukushima *et al.* 1992). The immune system uses these important substrates to support its activity. By this action, cytokines increase the resistance of the host against invaders and promote wound healing (Heinrich *et al.* 1990; Newsholme *et al.* 1985). On the other hand, it is worth mentioning that in mammals there is no protein storage depot (Tracey, 1994). Therefore, prolonged or inappropriate production of cytokines may lead to tissue damage, and prolonged wound healing.

In skeletal muscle, oxidation of branched-chain amino acids, namely, leucine, isoleucine, and valine is increased due to an inflammatory stimulus. These amino acids are deaminated, and the carbon skeletons are converted to tricarboxylic intermediates, which are the same as the intermediates formed during glucose and fatty acid catabolism. These intermediates enter the Kreb cycle to produce ATP, or can be used to make glucose or fat. The amine groups released from the above process are attached to pyruvate and glutamic acid to form alanine and glutamine respectively (Williamson, 1992). These products, with other amino acids reaching the blood stream, are taken up by the liver. Subsequently, they can be used to synthesise acute phase proteins or may be deaminated and the carbon skeleton enter the gluconeogenic pathway with subsequent excretion of the nitrogen in the form of

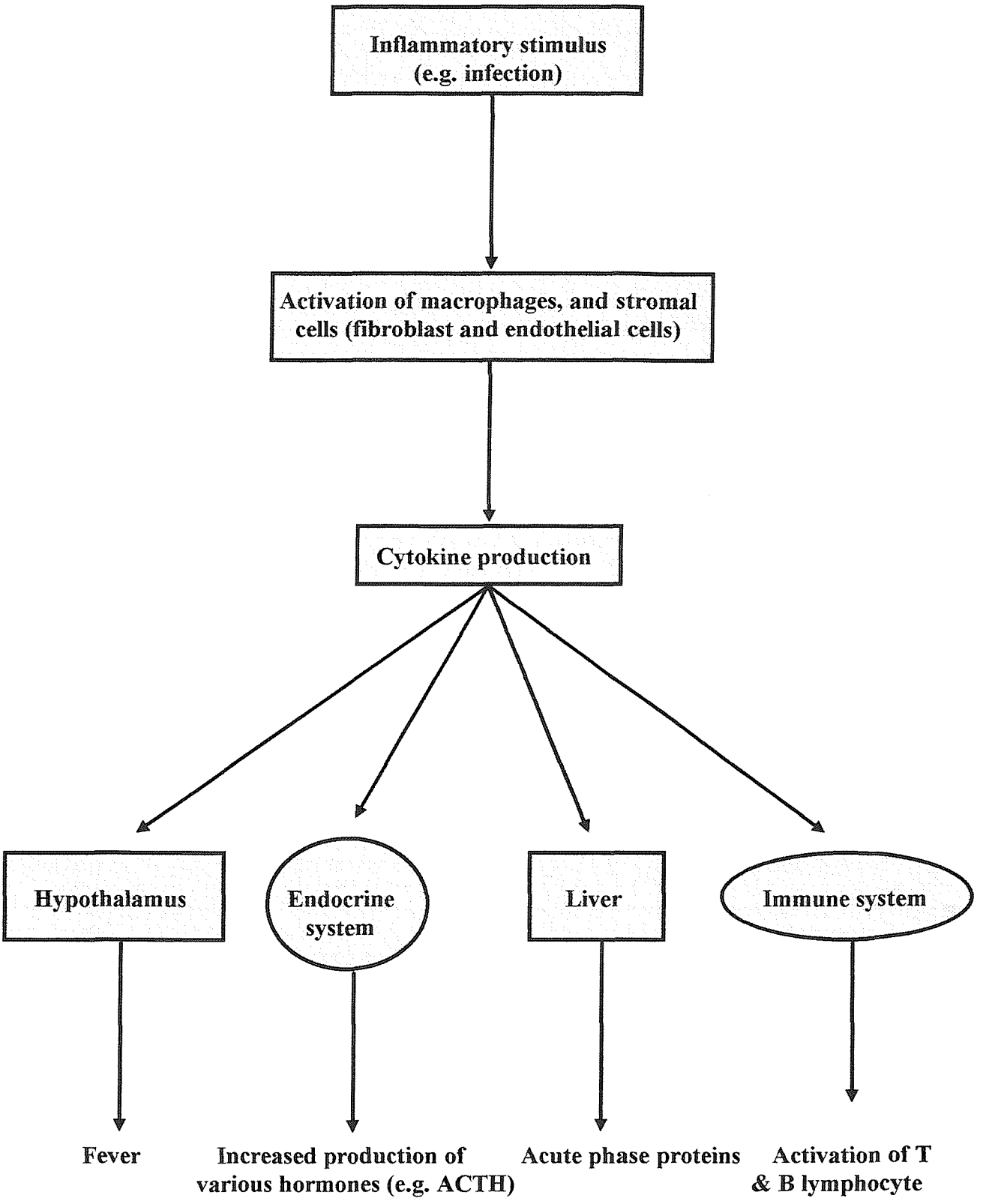
urea, ammonia, and other nitrogenous compounds in urine (Long *et al.* 1977; Beisel & Wannemacher, 1980; Jennings & Elia, 1996)

Glutamine released as a result of muscle breakdown and glucose synthesised in liver due to increase gluconeogenesis from aminoacids, are major fuel sources for the immune system (Newsholme *et al.* 1985; Newsholme & Newsholme, 1989).

1.3 Acute phase response

The acute phase response (APR) refers to the wide range of physiological changes executed by the host, immediately after infection or injury has occurred, in an effort to neutralise the inflammatory agent and activate the repair process. The APR is characterised by fever, changes in vascular permeability, increased production of various hormones including glucagon, adrenocorticotrophic hormone (ACTH), cortisol, glucocorticoids, thyroid stimulating hormone, and adrenaline, and the production of acute phase proteins. In case of injury, a number of additional responses such as aggregation of platelets and clot formation, dilation, leakage of blood vessels and activation of macrophages occur at the site of injury. These physiological changes are initiated by inflammatory mediators, which include cytokines, anaphylatoxins and eicosanoids. These mediators enhance the proliferation, and the biosynthetic activities of many cells and organs. Tissue macrophages or blood monocytes are the cells most commonly associated with initiating the cascade of events during the acute phase response. Activated macrophages release cytokines, such as IL-1 and TNF families, which are very important in initiating the series of reactions that occur immediately after exposure to an inflammatory stimulus (Figure 1-5). Furthermore, IL-1 and TNF, produced from macrophages, activate fibroblasts and endothelial cells, thus releasing other cytokines (reviewed in Baumann & Gauldie, 1994). The changes in body performance aim to protect the host, enhance the repair process, and restore the disturbed physiological homeostasis.

Figure 1-5 cascade of events during the acute phase response



Fever, one of the main signs of the acute phase response, is mostly initiated by the production of cytokines. IL-1 raises the hypothalamic set point by acting on neurones located in the preoptic area of the anterior hypothalamus, through increased production of prostaglandins, most notably prostaglandin E. The rise in the thermostatic set point drives mechanisms of heat conservation (vasoconstriction) and heat production (muscle shivering) until the blood and core temperature are elevated to match the hypothalamic set point (Dinarello & Wolff, 1982).

One of the important physiological signs of the APR is the dramatic alteration in the protein biosynthetic profile in the liver, which in turn cause changes in the concentration of some plasma proteins (Heinrich *et al.* 1990). These proteins are called the acute phase proteins.

1.4 Acute phase proteins

Acute phase proteins are those plasma proteins, synthesised predominantly by the liver, whose concentrations are altered during inflammation. These changes in the concentration of proteins can be positive or negative. Positive acute phase proteins are those proteins which increase their concentrations after inflammation (e.g. ceruloplasmin, C-reactive protein, α_1-acid glycoprotein, α-2 macroglobulin, haptoglobin, α1-proteinase inhibitor, and serum amyloid A protein). Negative acute phase proteins are those proteins which decrease their concentrations after inflammation such as albumin and transferrin (Fleck, 1989). Table 1.3 shows the concentrations of acute phase proteins during normal health and following an APR in humans.

Table 1.3 The plasma concentrations of some positive and negative human acute phase proteins under normal and acute phase response conditions reported from several studies

Acute phase protein	Normal value mg/ml	Acute phase value mg/ml
Serum Amyloid A	0.01	1.0-2.0[d]
α-1 acid glycoprotein	0.6-1.2	1.5-2.5 [a,b,c]
Ceruloplasmin	0.3-0.4	0.4-0.6[a,b,c]
α1-proteinase inhibitor	1.5-3.0	4.0-6.0 [a,b,c]
Haptoglobin	1.0-2.0	5.0-6.0 [a,b,c]
C-reactive protein	0.001	0.3-0.5[d]
Albumin	35-45	20-30 [a,b,c]
Transferrin	2.0-3.0	1.5-2.0 [a,b,c]

a; Allen *et al.* (1977), b; Lebreton *et al.* (1979), c; Putnam (ed.) (1975), d; Pepys & Baltz (1983).

As described earlier, the alteration in the hepatic protein synthesis is regulated by IL-6, IL-1 and TNF (Heinrich *et al.* 1990). IL-1 and TNF-α, produced during the APR, stimulate the synthesis of glucocorticoids located in the adrenal glands, via the central nervous system. Consequently, the increase production of glucocorticoids enhances the synthesis of acute phase proteins indirectly, by enhancing the effect of IL-1 and IL-6 on many of the acute phase proteins (Fey & Gauldie, 1990; Prowse & Baumann, 1988). On the other hand, glucocorticoids down-regulate the production of cytokines from macrophages (Beutler *et al.* 1986).

The acute phase proteins have a wide range of functions that aid in host defence. Some of the well-documented functions of acute phase response are listed in Table 1.4.

Table 1.4 Biological functions of some acute phase proteins

Acute phase proteins	Biological functions
α1-proteinase inhibitor[1] α1- antichymotrypsin[1] α2- macroglobulin[1]	Inhibition of proteinases
C-reactive protein[1] Serum amyloid A [1]	Removal of antigens from the host
C-reactive protein[2] C3 complement[1]	Activation of the immune response
Proteinase inhibitors[1,3] α1-acid glycoprotein[1,2]	Suppression of the immune response
Ceruloplasmin[1,4,5] α1-acid glycoprotein[6,7]	Behaving as an antioxidant
Ceruloplasmin[1] Haptoglobin[1] Transferrin[1] α1-acid glycoprotein[1,8]	Binding and transport of metals and biologically active compounds

1; Koj (1985b), 2; Goldstein & Elwyn (1989), 3; Carney *et al.* (1998), 4; Leung (1998), 5; Segelmark *et al.* (1997), 6; Costello *et al.* (1984), 7; Laine *et al.* (1990), 8; Moore *et al.* (1997).

The biological properties of C-reactive protein, one of the main acute phase proteins in human, and α-1 acid glycoprotein, one of the very strong acute phase proteins in rat (Heinrich *et al.* 1990) will be discussed in details below. These two acute phase proteins show the highest increase during an acute phase response, with serum levels elevated to 20-1000 fold at the onset of inflammation.

1.4.1 C-reactive protein

C-reactive protein (CRP) was originally named for its ability to bind and precipitate the C-polysaccharide fraction of pneumococcal lysates. CRP may increase up to 1000-fold during the acute phase response (Baltz *et al.* 1982). CRP opsonize bacteria for phagocytosis, and activates the classical complement pathway (Volanakis, 1982). CRP has the ability to bind and clear nuclear materials released from the cells due to the destruction of foreign particles (Robey *et al.* 1984). In the presence of calcium ions, CRP binds phosphorylcholine residues, and other microbial complex polysaccharides, and thus aid in their detoxification and clearance (Pepys & Baltz, 1983). Heurtz *et al.* (1996) have shown a reduction in the neutrophil infiltration in the bronchoalveolar lavage fluid after injection of CRP peptide to mice. This function of CRP was supported by another study conducted by Zouki *et al.* (1997) who found that CRP has the ability to diminish neutrophil infiltration at the site of inflammation by cleavage and shedding of selectin, a molecule necessary for the interaction of neutrophil with endothelial cells.

1.4.2 α-1 acid glycoprotein

α-1 acid glycoprotein, is a single polypeptide chain, with five carbohydrate (CHO) chains attached to it. It can be distinguished from other glycoprotein by it is high content of CHO (45%), including large amounts of sialic acid (Yoshima *et al.* 1981). It is produced mainly by liver, however, inflammatory cells such as lymphocytes, and polymorphonucleocytes (PMN) can also produce α-1 acid glycoprotein in response to an inflammatory stimulus (Gahmberg & Anderson, 1978). α-1 acid glycoprotein can be classified as a very strong acute phase reactant in rat, and a strong reactant in human (Koj, 1985a; Heinrich *et al.* 1990).

α-1 acid glycoprotein has been shown to inhibit platelet aggregation (Costello *et al.* 1979). Superoxide production ($O_2^{\bullet -}$) by neutrophils was inhibited in the presence of α-1 acid glycoprotein (Costello *et al.* 1984 & Laine *et al.* 1990). In Laine's study (1990), for example, PMN stimulated with opsonized zymosan or with phorbol myristate acetate show some inhibition of $O_2^{\bullet -}$ production after addition of

α-1 acid glycoprotein. This inhibition was dose related. Thus, α-1 acid glycoprotein may function as an antioxidant compound.

Reeds *et al.* (1994) suggest that the amino acid composition of skeletal muscle proteins does not match the amino acid requirement for synthesising acute phase proteins, during the acute inflammatory response. As a result of this mismatch, between the amino acids released from muscle and requirement of acute phase proteins, some amino acids will be released in quantities surplus to these requirements. The surplus amino acids would be catabolised contributing to the negative nitrogen balance, which is characteristic of the infected traumatised individual. This hypothesis is discussed in further detail later, and is investigated in the thesis using rats as the experimental model. Table 1.5 shows that the amino acid composition of rat muscle protein was quite similar to the amino acid composition of human muscle protein. However, Reeds *et al.*'s (1994) hypothesis was not based on the amino acid composition of human muscle protein, but it based on the mean values of amino acid composition of bovine, porcine and ovine muscle proteins.

Table 1.5 The amino acid composition of muscle proteins (g amino/kg protein)

Amino acid	Rat muscle protein[1]	Human muscle protein[1]	Mammal[2]	Mean values of bovine, porcine and ovine muscle[3]
Arginine	45	41	66	69
Histidine	40	32	28	51
Isoleucine	35	38.5	47	48
Leucine	102	102.5	80	81
Lysine	90	85.5	85	98
Methionine	21	20.5	25	25
Phenylalanine	75	74.5	45	40
Threonine	77	70	46	47
Valine	71	73.5	55	54
cystine	14	17.5	14	13
tryptophan	18	21	11	13
alanine	65	65.5	65	59
aspargine + Aspartate	50	56.5	80	92
glutamine + glutamate	135	139	146	145
glycine	38	33.5	50	45
Proline	40	73.5	50	48
serine	86	74	51	41
tyrosine	28	28	31	36

[1] Mean values of rat and human muscle proteins taken from Block *et al.* (1956).
[2] Mean values of beef, human, lamb, rabbit, rat, swine, pork, and veal muscle proteins taken from Block *et al.* (1956).
[3] Reeds *et al.*'s (1994) study had based their calculations on the mean values of bovine, porcine and ovine muscle proteins taken from Anderson *et al.* (1986).

1.5 Infection and growth

Infectious diseases of childhood are usually associated with decreased weight gain and linear growth (height) (Stephensen, 1999). Pathogens affect the growth process by reducing food intake and by changing the partitioning of nutrients away from peripheral tissues (e.g. skeletal muscle) toward immune cells and visceral tissues (e.g. liver). It is now well accepted that the deterioration in growth, which

results from loss of tissue lipid, protein, and micronutrients, is partly due to the induction of cytokines, particularly IL-1, IL-6, and TNF, in response to inflammatory stimuli. Nutrients released from peripheral tissues are used in metabolic responses that nourish the immune system and protect the host. As indicated earlier (section 1.2.1) the metabolic effect of IL-1, IL-6 and TNF can be direct via interaction with tissues, and indirect via stimulating the central nervous system (Grimble, 1994a; Johnson, 1997).

Receptors for IL-1 and TNF-α were found in brain tissue (Parnet *et al.* 1994; Kinouchi *et al.* 1991). In addition to that, brain cells such as astrocytes and microglia are able to synthesise IL-1, IL-6 and TNF-α (Benveniste, 1992). The stimulation of the central nervous system by cytokines increases the activity of the sympathetic nervous system and the formation of corticotrophin, and thus increases the production of counter regulatory hormones (CRHs) (Cortisol, glucagon, adrenaline and growth hormone). CRHs increase the breakdown of peripheral tissue, providing free CHO, fat, protein, and micronutrients to the immune system (Grimble, 1990).

When the induction of cytokines was initiated by injection of LPS in rats, protein synthesis in muscle was decreased by 30 to 40%, after 24 hours of giving LPS. The reduction in protein synthesis cannot be explained solely by the reduction in food intake. Thus, anorectic LPS-treated rats experience a greater fall in muscle protein synthesis compared with fasted-control rats. On the other hand, hepatic protein synthesis and hepatic protein content was markedly elevated in LPS-treated animals compared with control or fasted rats (Jepson *et al.* 1986).

TNF has been shown to increase lipolysis and inhibit lipoprotein lipase activity in peripheral tissue (Memon *et al.* 1994). Bone growth might also be affected by the production of cytokines. Reduction in osteoblast proliferation and osteoclast production occurred by increasing production of IL-1 and TNF-α (Skerry, 1994). When experimental colitis was induced in rats, suppression of bone growth occur, and the decrease in linear bone growth was positively correlated with the

level of IL-6 (Koniaris, 1997). Furthermore, Saklatvala (1986) and Black *et al.* (1990) have shown that TNF or IL-1 administration accelerates bone resorption.

Cytokines may also be involved in the anorexia found in infected patients, either directly by affecting the appetite centres by affecting the firing rate of glucose-sensitive neurones in the lateral hypothalamus (Plata-Salaman *et al.* 1988), or indirectly by increasing the formation of corticotrophin-releasing factor (Uehara *et al.* 1989). The three major findings obtained from Uehara's study, an in vivo study conducted in rats, were:

1) The administration of either LPS, a potent stimulator of the production of IL-1, or IL-1, both caused a reduction in food intake in a dose-related manner. The reduction in food intake induced by IL-1 was similar to those induced by LPS.

2) When prednisolone, a glucocorticoid hormone, which has been shown to inhibit cytokine production, was injected to the LPS-treated rats, the magnitude of reduction in food intake was markedly suppressed.

3) Finally, the reduction in food intake found after IL-1 administration was completely prevented when hypothalamic CRF was immunoneutralised by intracerebro-ventricular injection of rabbit CRF antiserum.

To conclude, cytokines are involved in the deterioration of growth usually seen in infected individual. This occurs by causing anorexia, increasing peripheral tissue breakdown, and by diverting nutrients towards immune cells at the expense of growth process.

1.6 Free radicals, oxidant molecules and antioxidant defences

1.6.1 Free radicals and oxidant molecules

Free radicals are molecular intermediates that have one or more unpaired electron, and arise during oxidation reactions. They are capable of accepting electrons and abstracting hydrogen ions from other molecules with which they come into contact, causing structural and functional damages. Some internal and external sources of free radicals are shown in Table 1.6. These molecules are highly reactive

and readily attack other molecules forming more free radicals in a chain reaction. Reactive oxygen species (ROS) is a general scientific term usually used to refer to free radical molecules, and to non-radical molecules that are able to oxidised other biomolecules, or are easily converted into radicals (Halliwell, 1996). Table 1.7 shows some important ROS found in aerobic-living organisms.

Table 1.6 Some sources of free radicals

Internally generated sources
Mitochondria
Phagocytes
Xanthine oxidase
Reactions involving iron and other transition metals
Arachidonate pathways
Peroxisomes
Exercise
Inflammation
Ischaemia / reperfusion
External sources
Cigarette smoke
Environmental pollutants
Radiation
Ultraviolet light
Certain drugs, pesticides, anaesthetics, and industrial solvents
Ozone

***Source: Langseth, 1995 (ILSI Europe Concise Monographs).**

Table 1.7 Name and structure of some reactive oxygen species found in aerobic-living organism

Name	Structure
1- Free radicals	
a) Hydroxyl radical	**$OH^{\bullet}$**
b) Superoxide radical	**$O_2^{\bullet -}$**
c) Lipid peroxyl radical	**$LOO^{\bullet}$**
d) Nitric oxide radical	**$NO^{\bullet}$**
2-Non radical-oxidant molecules	
a) Hydrogen peroxide	**H_2O_2**
b) Hypochlorous acid	**HOCl**
c) Ozone	**O_3**

1.6.2 Oxidation of biomolecules by oxygen radicals

Since we are oxygen (O_2)-dependent hosts, the most important biological radicals, are the O_2 radicals. Molecular O_2 is a radical molecule, having two unpaired electrons with a parallel spin configuration, donated by  .

Most other biomolecules are covalently bonded non radicals, having pairs of electrons with an opposite spin configuration  . Therefore, the reaction of O_2 with biomolecules is spin restricted. The difference in spin configuration would prevent the O_2 from accepting a pair of electrons at once, or simultaneously. Thus it slows down the reaction of O_2 with biomolecules. Furthermore, the spin feature of O_2 allows this molecule to be a one-electron reductant molecule,  . The acceptance of one electron lead to the formation of superoxide radical (reviewed in Halliwell & Gutteridge, 1990).

1.6.3 Sources of reactive oxygen species

1.6.3.1 The production of reactive oxygen species as a consequence of normal metabolic processes

ROS such as superoxide ($O_2^{\bullet -}$), and hydrogen peroxide (H_2O_2) can be continuously produced inside human body, as a consequence of normal metabolic process. There are many molecules that are unstable in the presence of O_2 and undergo oxidation reactions. Halliwell, gives a good example, he said, and I quote "If a solution of the hormone epinephrine (adrenaline) is left standing on a laboratory bench, it becomes oxidised. It undergoes a chemical reaction with O_2 , making $O_2^{\bullet -}$ and H_2O_2" (Halliwell, 1997). Thus, logically, our molecules are at risk of undergoing oxidation reactions. Some examples of non-enzymatic and enzymatic free radicals reactions can be seen in Figure 1-6. Another way of producing ROS, is by the electron transport chain (ETC) in the mitochondria. There are a small percentage of electrons that escape from the electron carriers in the ETC and react with O_2 making $O_2^{\bullet -}$ and H_2O_2. These molecules, however, are not highly reactive,

but if they come in contact with transition metals, such as iron and copper, they will be converted to the highly reactive hydroxyl radical ($OH^{\bullet}$).

Figure 1-6 Examples of free radicals reactions

1) Enzymatic production of free radicals

a) $Xanthine + O_2 + H_2O \xrightarrow{\text{Xanthine oxidase}} NADP^+ + H^+ + 2O_2^{\bullet -}$

b) $NADPH + 2\ quinone \xrightarrow{\text{Cytochrome p450 reductase}} NADP^+ + 2\ semiquinone^{\bullet} + H^+$

2) Non enzymatic production of free radicals

a) $O_2 + Fe^2 \longrightarrow Fe^3 + O_2^{\bullet -}$

b) $H_2O_2 + Fe^2 \longrightarrow Fe^3 + OH^{\bullet} + OH^{\bullet}$

1.6.3.2 Enhanced production of reactive oxygen species as part of immune defence

ROS can also be produced at enhanced rate as a part of defence mechanism. When phagocytes or neutrophils come in contact with a foreign particle, they produced $O_2^{\bullet -}$ and H_2O_2 with the purpose of inactivating or destroying the antigen. $O_2^{\bullet -}$, H_2O_2, $OH^{\bullet}$, and hypochlorous acid (HOCl) can be produced when phagocytic cells are activated (Droge *et al.* 1994; Halliwell *et al.* 1988). Neutrophils, one of the major circulating PMN, account for 70% of total PMN circulating in the blood. When they come into contact with a foreign particle, the enzyme NADPH oxidase, located in the plasma membrane, is activated. Once the enzyme is activated, NADPH, found in the cytoplasm of neutrophils, is converted to NADP, and the electrons passed onto O_2, outside the cell, converting it to $O_2^{\bullet -}$. The process of the rapid consumption of molecular oxygen that accompanies formation of $O_2^{\bullet -}$ is called the 'respiratory burst'. This process also occurred in other phagocytic cells

(monocytes, macrophages, and eosinophils). $O_2^{\bullet-}$ generating plasma membrane engulfs the particle, forming phagocytic vacuole, which then enters the cytoplasm of the neutrophil. Next, $O_2^{\bullet-}$ is converted to H_2O_2 in a non enzymatic reaction. The rate of this conversion is greatly increased by superoxide dismutase (Halliwell *et al.* 1988):

Reaction (1)

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{\text{Superoxide dismutase}} H_2O_2 + O_2$$

Neutrophils use H_2O_2 to produce the highly reactive oxygen species $OH^\bullet$ and HOCl, which are highly toxic to foreign organisms. In the presence of trace amounts of iron and other transition metals, H_2O_2 can be converted to $OH^\bullet$:

Reaction (2)

Transition metals (reduced form) + H_2O_2 ⟶ $OH^\bullet$ + $OH^\bullet$ + Transition metals (oxidised form).

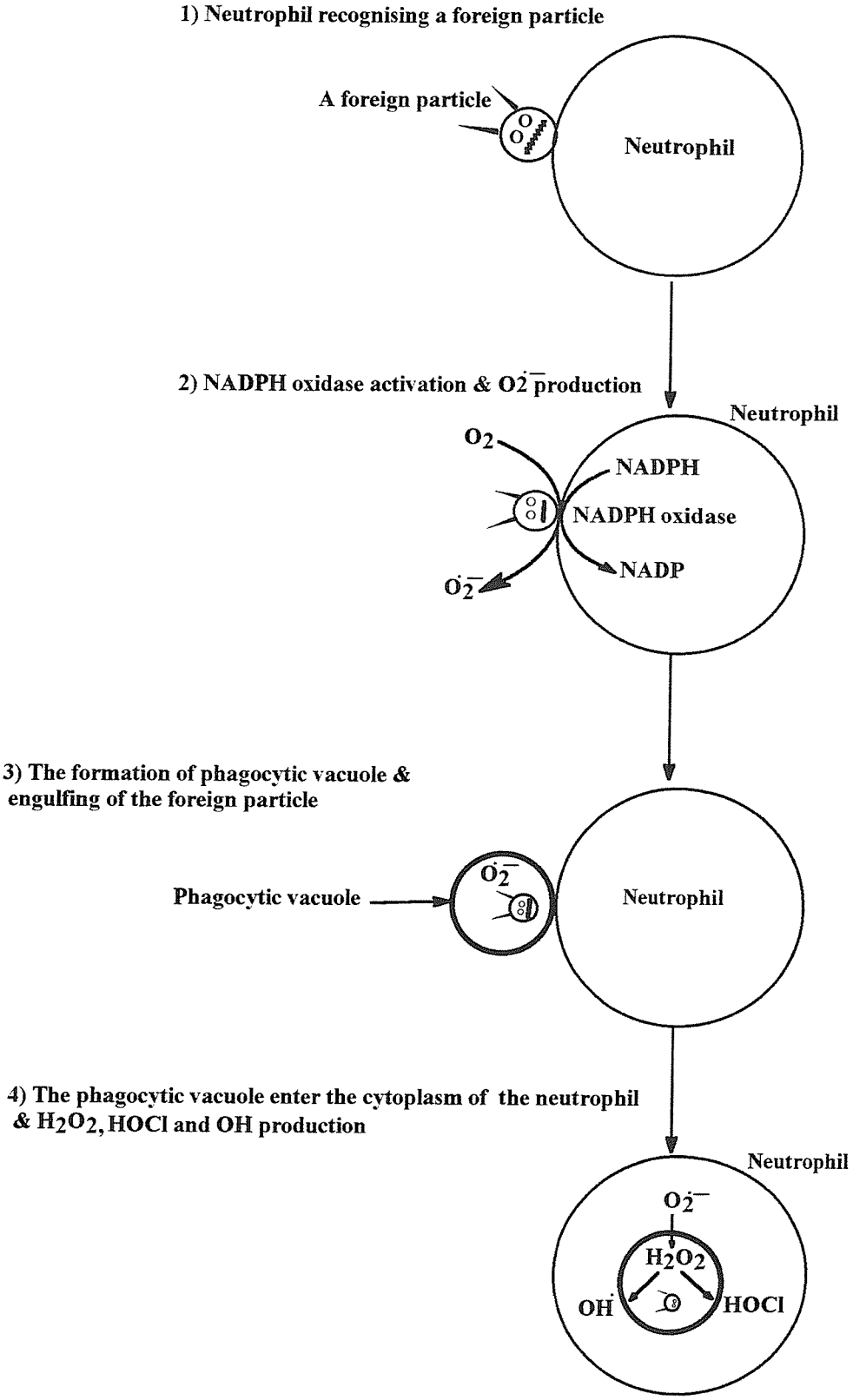
or by the presence of the enzyme myeloperoxidase, H_2O_2 is converted to HOCl (HOCl; the major component of household bleach that kills bacteria):

Reaction (3)

$$H_2O_2 + Cl^- \xrightarrow{\text{Myeloperoxidase}} HOCl^- + H_2O$$

Figure 1-7 highlights the important steps of the production of ROS by neutrophils.

Figure 1-7 The production of reactive oxygen species from neutrophils



1.6.4 Possible side effects caused by overproduction of reactive oxygen species

The advantages of producing ROS are to kill invading pathological organisms, destroy damaged tissue, and enhanced wound healing by stimulating fibroblast proliferation. However, these molecules carry the risk of destroying the host tissues. When, for example, $OH^{\bullet}$ attacks polyunsaturated fatty acids (PUFA), located in the cell membrane, it abstracts an atom of hydrogen to form water. Is the process of further damage stopped by converting $OH^{\bullet}$ to water? Unfortunately, the answer is no. PUFA with unpaired electrons on a carbon atom, after an electron has been removed, undergoes molecular arrangements to form a conjugated diene. The conjugated diene react with oxygen to form a peroxy radical. Peroxy radicals can abstract hydrogen atoms from another fatty acid side-chain to form lipid peroxides (Halliwell, 1987). Consequently, the radical chain reaction of abstracting hydrogen and reacting with oxygen continues (Figure 1-8 shows the steps of lipid peroxidation). Damage to protein can be caused by the production of ROS. Inactivation of protease inhibitors, such as α-1 protease inhibitor, may be caused by the production of HOCl. ROS are also able to damage deoxyribonucleic acid (DNA), forming DNA base adducts (Elghissassi *et al.* 1995). DNA-repairing enzymes and polymerases can be extensively damaged by oxidant molecules (Wiseman & Halliwell, 1996). $OH^{\bullet}$ is able to attack all four DNA bases, while $O_2^{\bullet -}$ and H_2O_2 are unable to react with DNA bases (Halliwell & Aruoma, 1991). Other types of possible damage caused by free radicals can be seen in Figure 1-9.

Although the phagocytic defence mechanism is beneficial to the body, over or unregulated production of ROS, from inappropriate activation of phagocytic cells, can damage host cells. Many chronic inflammatory diseases, such as inflammatory bowel disease and rheumatoid arthritis are accompanied by sustained production of ROS, due to inappropriate phagocyte activation (Grisham, 1994 & Halliwell, 1995). The continuous production of ROS would therefore overwhelm the antioxidant defences, and damage tissue.

Figure 1-8 Lipid peroxidation

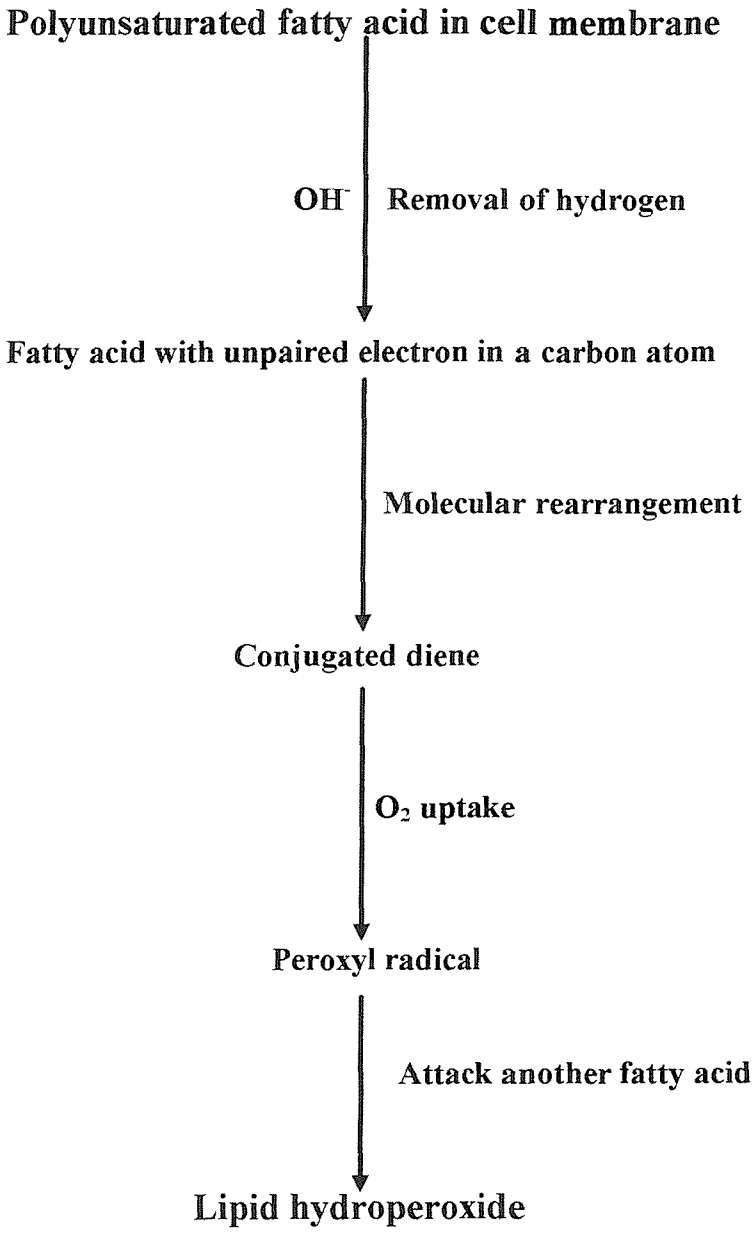
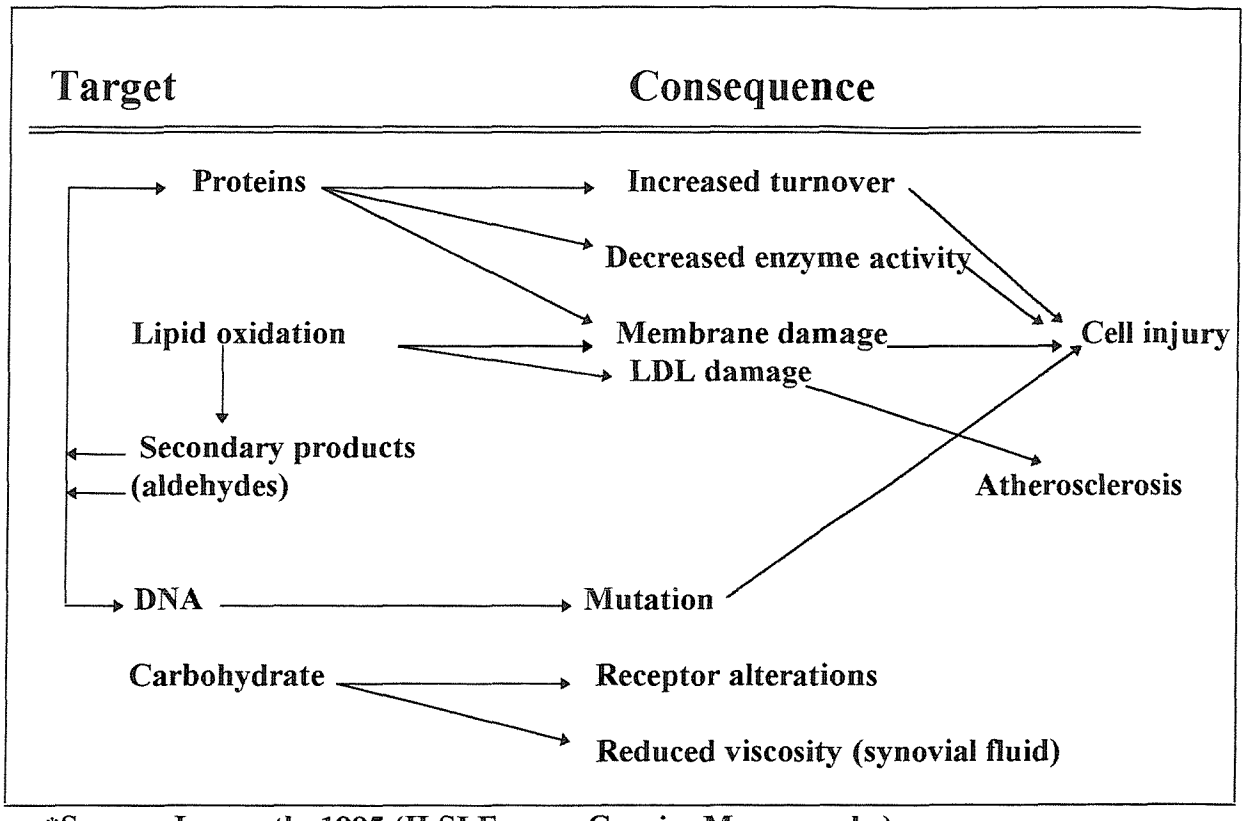


Figure 1-9 Free radical damage



***Source: Langseth, 1995 (ILSI Europe Concise Monographs).**

The risk produced from these free radical molecules can be minimised if there are enough antioxidant defences to stop the oxidant chain reactions. There are a variety of antioxidant molecules and enzymes e.g. vitamins E & C, GSH, GSH peroxidase, catalase, and superoxide dismutase, which can protect mammalian tissues from free radicals by converting them into non-harmful molecules. For example, vitamin E has the ability to break the chain of lipid peroxidation by donating the hydrogen atom of the hydroxyl group on its chromanol ring to the free radical and convert it to a stable molecule (Garrow & James, 1993). Superoxide dismutase catalyses the removal of the toxic superoxide radical anion to a molecular oxygen and hydrogen peroxide (see reaction 1, p 28).

Hydrogen peroxide can either be rapidly converted to water by the action of the enzyme catalase:

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

Or in the presence of GSH, which is a very important antioxidant in the body, the enzyme GSH peroxidase has the ability to remove H_2O_2:

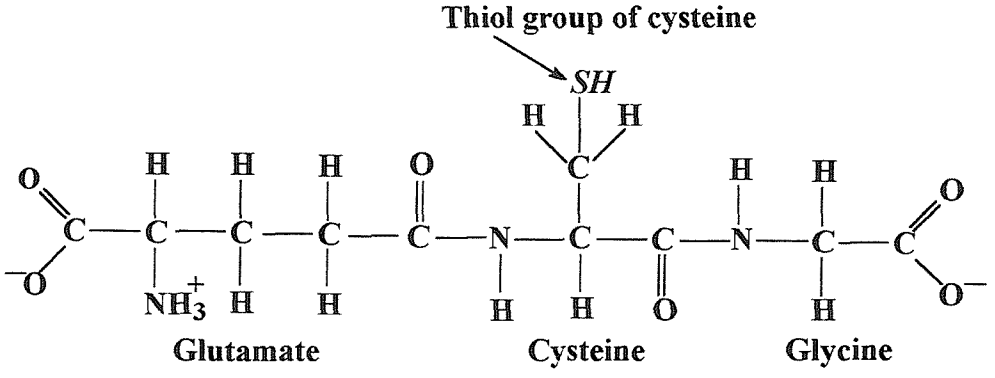
2GSH (reduced form) $+2H_2O_2$ $\xrightarrow{\text{GSH peroxidase}}$ GSSG (the oxidised form of GSH) + $2H_2O$

It is worth mentioning that GSH peroxidase is more important in scavenging H_2O_2 than catalase. This is because GSH peroxidase is found in mitochondria and cytosol, and has the same location of superoxide dismutase, while catalase is located in peroxisomes (Halliwell, 1987).

1.7 Glutathione-cycling and function

Glutathione (GSH), one of the major antioxidant compounds in tissue, was named philothion by de Rey-pailhade in 1888, which is a Greek word means; philo=love, thion=sulphur. In 1929, Sir Frederick Gowland Hopkins discovered that philothion is actually a thiol compound, and it was renamed glutathione (GSH). GSH consists of three amino acids, cysteine, glycine, and glutamate (Figure 1-10), in which cysteine is the most limiting amino acid for glutathione synthesis (Meister *et al.* 1986).

Figure 1-10 The structure of Glutathione



GSH has three important structural characteristics without which its metabolic and functional properties would be abolished. The three structural characteristics are:

1) The presence of the thiol group (SH) of cysteine, which is actually the major structural criterion of GSH, bestowing GSH with most, if not all of its metabolic functions.

2) Cysteine is bound to the γ-carboxyl group of glutamate instead of the common α-carboxyl group. This distinct criterion allows γ-glutamylcysteine, an intermediate in the GSH metabolic pathway, to be resistant to all dipeptidases, except γ-glutamyl transpeptidase.

3) The presence of glycine protects GSH from cleavage by the intracellular enzyme, γ-glutamylcyclotransferase.

Most of glutathione present in cells is in the form of GSH, and more than 90% of non-protein sulphur of the cells is in the form of GSH (Meister, 1995). The liver is the major site of GSH synthesis and transport (Lauterburg *et al.* 1984). Other organs such as kidney, lung, spleen, intestine and thymus are also capable of synthesising GSH (Meister, 1989).

Two consecutive steps are required to synthesise glutathione, each step consumes one ATP molecule:

1) L-Glutamate + L-cysteine + ATP $\xrightarrow{\textbf{γ-glutamylcysteine synthetase}}$ γ-glutamyl-cysteine + ADP + Pi

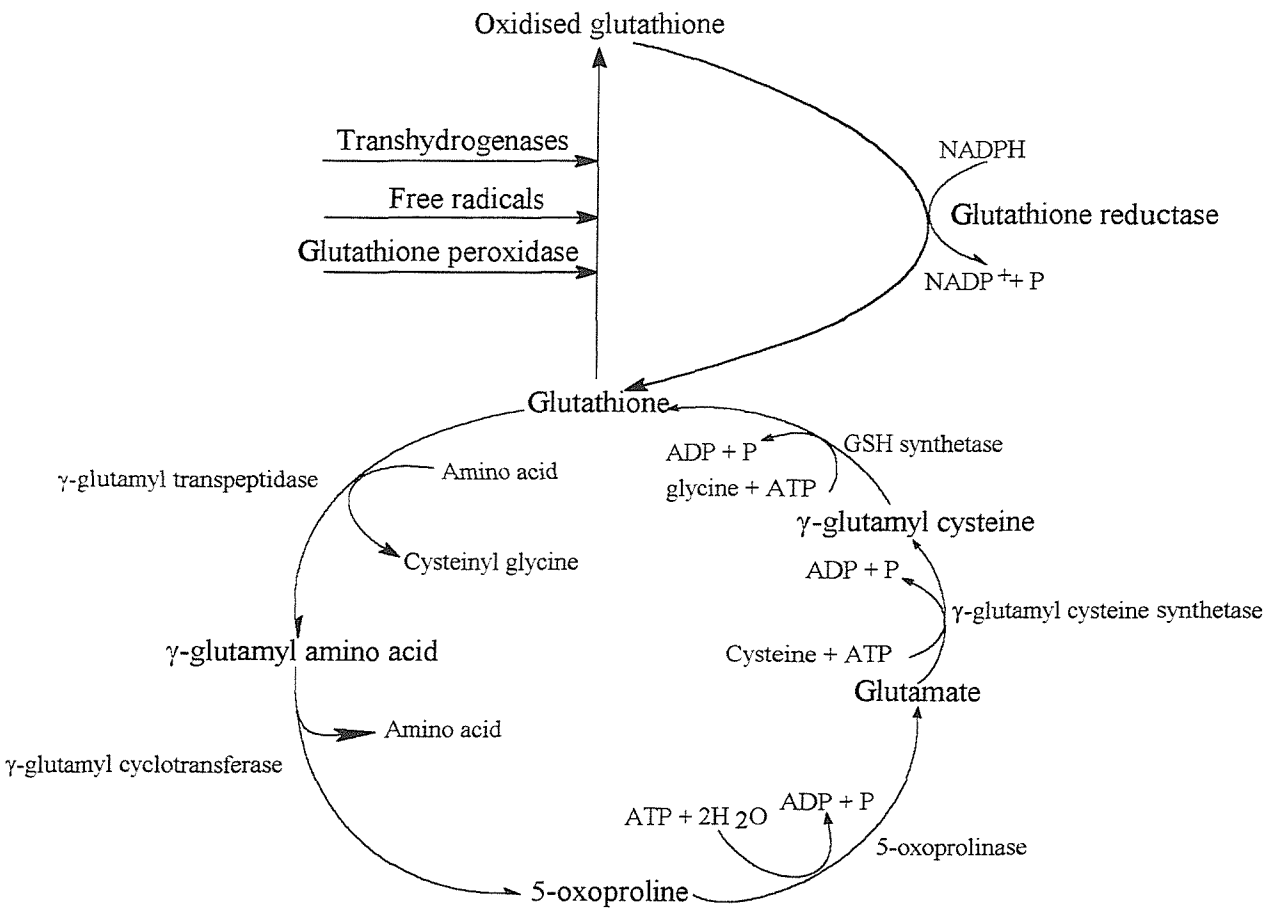
2) γ-glutamyl-cysteine + glycine + ATP $\xrightarrow{\textbf{GSH synthetase}}$ GSH + ADP + Pi.

The rate-limiting enzyme in the pathway for GSH synthesis is γ-glutamylcysteine synthetase. Under normal physiological conditions the activity of γ-glutamylcysteine synthetase is feed back inhibited by GSH (Richman & Meister 1975; Huang *et al.* 1993).

Since the enzymes involved in the degradation of glutathione, γ-glutamyl transpeptidase and dipeptidase are bound to the external surfaces of cell membrane (Tate & Meister, 1981), the degradation of glutathione occurs extracellularly.

Figure 1-11 illustrates the pathway of glutathione metabolism. By the action of γ-glutamyl transpeptidase, glutathione is broken down to γ-glutamyl amino acid and cysteinyl glycine (Allison & Meister, 1981). Cystine, methionine, glutamine, and many dipeptidases, especially aminoacyl glycine, are good acceptors of the above transpeptidation reaction (Thompson & Meister, 1975; Tate & Meister, 1974; Meister & Anderson, 1983).

Figure 1-11 Glutathione metabolism



γ-glutamyl amino acid is transported into certain cells. γ-glutamyl amino acid is substrate of the γ-glutamyl cyclotransferase, an intracellular enzyme which converts γ-glutamyl amino acid into 5-oxoproline and the corresponding amino acid. In an ATP dependent reaction, the enzyme 5-oxoprolinase converts 5-oxoproline to glutamate.

Exported GSH, and extracellular cystine interact with γ-glutamyl transpeptidase leading to the formation of γ-glutamyl cystine. γ-glutamyl cystine is transported into the cell, and reduced to form cysteine and γ-glutamyl cysteine. Cysteine and γ-glutamyl cysteine are substrates for γ-glutamyl cysteine synthetase and GSH synthetase, respectively.

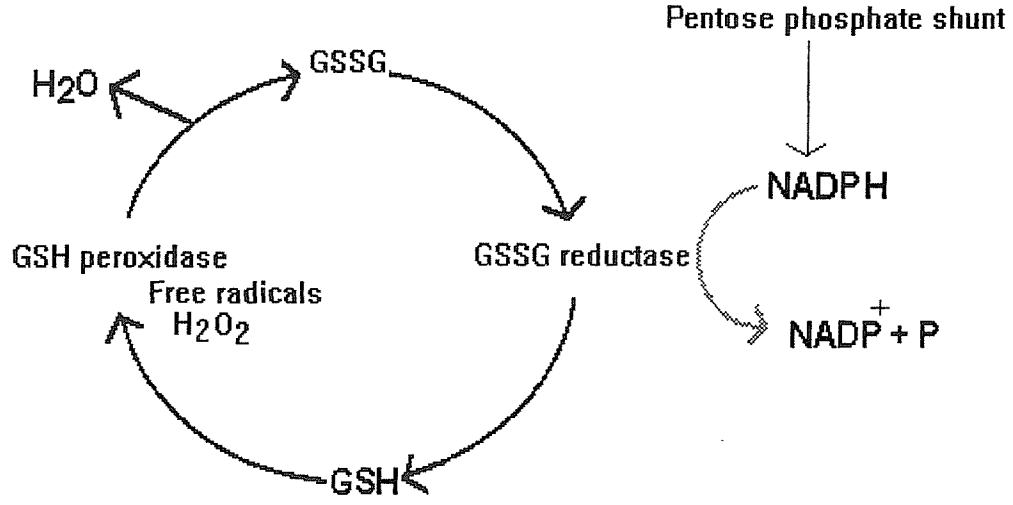
The liver is the major site for the synthesis of plasma GSH (Lauterburg *et al.* 1984). The transport of hepatic GSH to plasma is a way of distributing cysteine and GSH to other cells, after reacting with γ-glutamyl transpeptidase, which is located in the external surfaces of cell membranes. The role of the γ-glutamyl transpeptidase in utilising GSH has been shown by Meister and his colleagues (Griffith & Meister, 1979; Anderson *et al.* 1980). They found a dramatic increase of plasma GSH levels and glutathionuria after administering γ-glutamyl transpeptidase inhibitors to experimental animals, indicating the important of this enzyme in the cellular uptake and utilisation of plasma GSH.

GSH plays important functions within the cells. It is used by several transhydrogenases, and serves as a reductant by providing the H^+ to maintain other compounds in a reduced form (e.g. ascorbate, α tocopherol, coenzyme A and, deoxyribonucleotide). GSH serves as an antioxidant compound by reacting directly with free radicals or as a substrate for GSH peroxidase. NADPH is required for the conversion of the oxidised form of glutathione to the reduced form, which has the antioxidant property, in a reaction catalysed by glutathione reductase (Figure 1-12). GSH also serves as a storage form of cysteine (Meister, 1995).

It's worth mentioning that the antioxidant property of GSH is not only important in conditions where there is external source of oxidant agents, but also

under normal conditions. Mitochondria produce hydrogen peroxide from oxygen under normal physiological conditions (Boveris & Chance, 1973). Thus, GSH deficiency leads to damage of various tissues in mice and rats without administration of oxidant agents (Meister, 1991). Since mitochondria do not contain catalase (Meister, 1991), they depend mostly on GSH for scavenging oxidant molecules. It is also interesting to note that mitochondria do not have glutathione synthetic enzymes (γ-glutamyl cysteine synthetase and GSH synthetase). Thus, mitochondria fully dependent on GSH synthesised in the cytosol, which is then transported to mitochondria (Griffith & Meister, 1985).

Figure 1-12 Glutathione cycle



GSH also function as an immunomodulator. It is well established that an adequate amount of GSH is important for T-cells to function properly (Gmunder *et al.* 1990; Robinson *et al.* 1993; Fidelus & Tsan, 1986). For example, Gmunder *et al.* 1990 have shown that lymphocytes with high GSH level had increased DNA

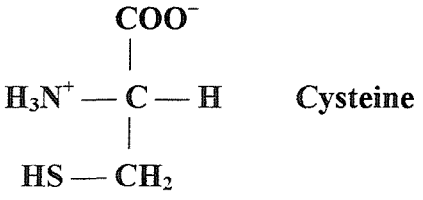
synthesis compared with lymphocytes with lower GSH level. However, the relationship between GSH and lymphocyte is complex. For example, in healthy human subjects, the number of $CD4^+$ T cells increases with intracellular GSH levels up to 20-30 nmol/μg protein, but declines at higher concentrations. A similar pattern also occurred with the number of $CD8^+$, in relation to GSH concentration, but to a lesser extent than $CD4^+$ (Kinscherf *et al.* 1994).

1.8 Sulphur amino acids (cysteine & methionine)

1.8.1 The chemical structure and sources of cysteine

Cysteine is a polar, uncharged sulphur amino acid (Figure 1-13).

Figure 1-13 The chemical structure of cysteine



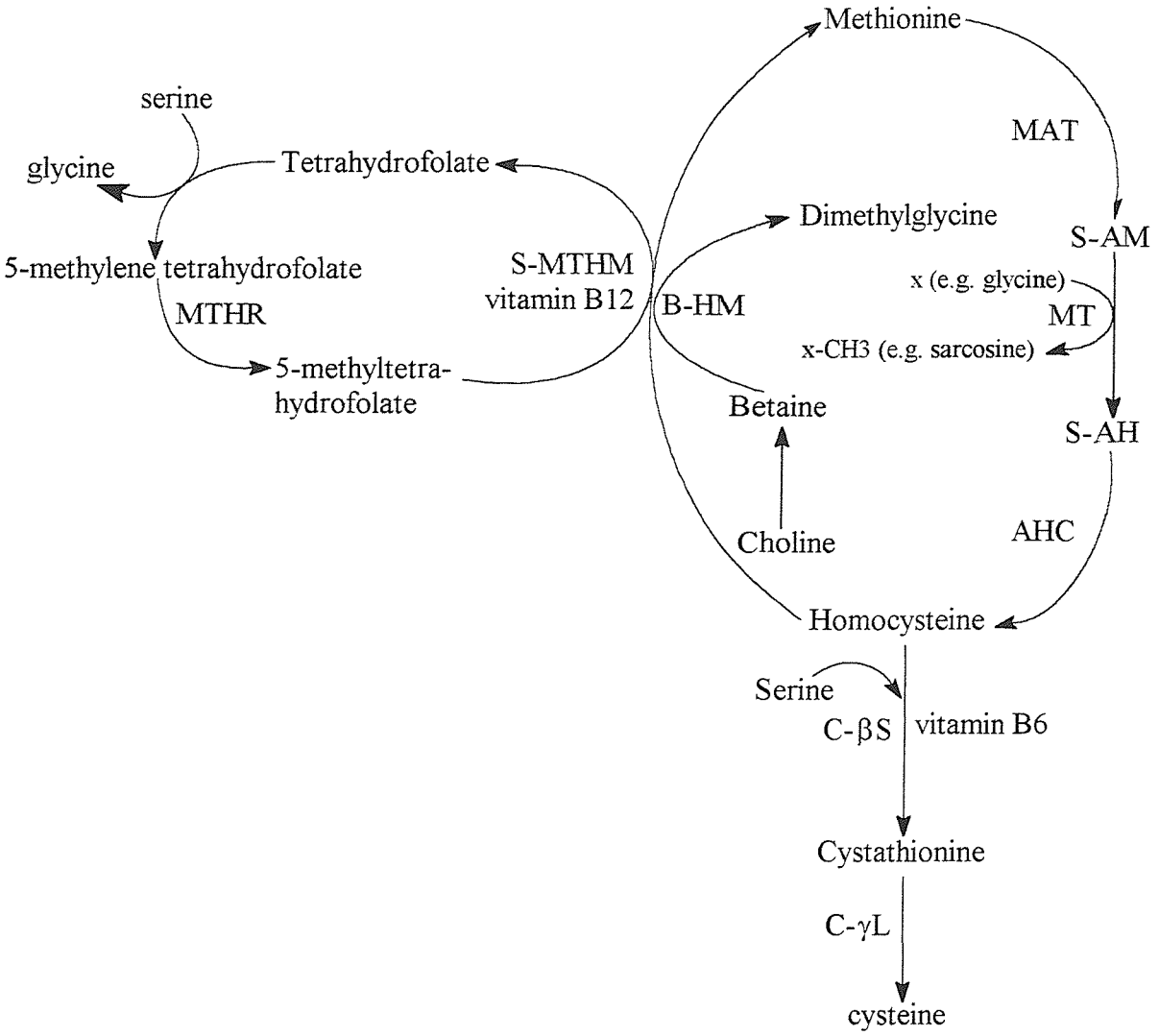
Cysteine

The most important source of cysteine is dietary intake (Bauman *et al.* 1988). Cysteine from dietary intake can be derived directly as an amino acid, or indirectly from methionine.

1.8.2 Cysteine synthesis from methionine

Cysteine can be synthesised from methionine via the transmethylation-transsulphuration pathway (Figure 1-14). Traver & Schmidt (1939) isolated ^{35}S-labelled cysteine from rat hair, after feeding ^{35}S-labelled methionine, indicating the transfer of methionine sulphur to cysteine sulphur.

Figure 1-14 The transmethylation-transsulphuration pathway



Keys: MAT: Methionine adenosyltransferase; S-AM: S-adenosylmethionine; MT: Methyl transferase; X: any methyl acceptor; S-AH: S-adenosylhomocysteine; AHC: Adenosyl homocysteinase; MTHR: Methylenetetrahydrofolate reductase; B-HM: betaine-homocysteine methyltransferase; S-MTHM: S-methyl tetrahydrofolate homocysteine methyltransferase; C-βS: Cystathionine β-synthase; C-γL: Cystathionine γ-lyase.

The first step in the transmethylation pathway starts when methionine is condensed with ATP in a reaction catalysed by methionine adenosyltransferase. S-adenosyl methionine (S-AM) is formed. S-AM transfers the methyl group to any methyl group acceptor such as guanidino acetic acid, in a reaction catalysed by methyltransferase, leaving S-adenosyl homocysteine, which is degraded by hydrolysis to form homocysteine, in a reaction catalysed by adenosyl homocysteinase. Homocysteine can either be methylated by betaine-homocysteine methyltransferase (B-HM), or by S-methyl tetrahydrofolate homocysteine methyltransferase (S-MTHM) to form methionine. The other possibility is to condense with serine, the first step in the transsulphuration pathway, in an irreversible reaction, catalysed by cystathionine β-synthase (C-βS) to form cystathionine. Due to this irreversible reaction, cysteine cannot be used as a precursor for methionine (Finkelstein, 1990). Greenberg (1975) demonstrated that cysteine synthesis from methionine via cystathionine is the sole pathway for the formation of cysteine in vertebrates. Hydrolytic cleavage of cystathionine, by the action of cystathionine γ-lyase, forms cysteine and α-ketoglutarate (reviewed in Horton *et al.* 1992 & Murray *et al.* 1988). Thus the enzymes S-MTHM and B-HM compete with the enzyme C-βS for homocysteine within the cell and determine whether methionine is recycled or committed to the transulphuration pathway. Because methionine is an essential amino acid for mammals, the availability of homocysteine for the synthesis of cysteine depends on an ample supply of methionine.

The Km values for the homocysteine methyltransferases enzymes, the methionine conserving enzymes, are two order of magnitude lower than cystathionine synthase and cystathionase, the homocysteine catabolising enzymes (reviewed in Finkelstein, 1990). Therefore, at low concentration of substrate, remethylation has a kinetic advantage compared to transsulphuration, and methionine will be conserved at the expense of cysteine synthesis. In this situation GSH synthesis would be compromised since cysteine is the rate limiting substrate in its formation.

Finkelstein & Martin (1984) have observed that in rats on a normal-protein diet 46% of homocysteine is converted to cystathionine, and 54% is remethylated to methionine, while in a low-protein diet only 25% of homocysteine is converted to cystathionine and 75% is remethylated to methionine. When the activity of C-βS, the enzyme necessary for condensing homocysteine with serine to form cystathionine in the first step of the transsulphuration pathway, was determined in rats given either 1% or 0.25% methionine with various amounts of cysteine, the addition of cysteine caused a reduction in the activity of C-βS, mainly by decreasing its mRNA level, indicating that a feedback system for cysteine synthesis occurs (Yamamoto *et al.* 1995). Thus cysteine synthesis is influenced by two major biochemical factors i.e. between S-MTHM, together with B-HM, and C-βS, and the feedback mechanism in GSH synthesis; and on a nutritional factor, the amount of sulphur amino acid consumed.

In a survey of the enzymes of sulphur amino acid metabolism in the rat, Finkelstein (1990) showed that all tissues contained methionine adenosyltransferase, adenosyl homocysteinase, methyltetrahydrofolate reductase, methyltetrahydrofolate homocysteine methyltransferase. Only liver contained betaine-homocysteine methyltransferase, and five organs lacked cytathionine synthase (spleen, adrenal, lung, testes and heart). From this profile of enzymes it could be concluded that only liver can totally recycle methionine, and that spleen, adrenal, lung, testes and heart are incapable of synthesising cysteine, and must therefore extract the amino acid from the blood stream either as such or in the form of GSH.

1.8.3 Cysteine metabolism

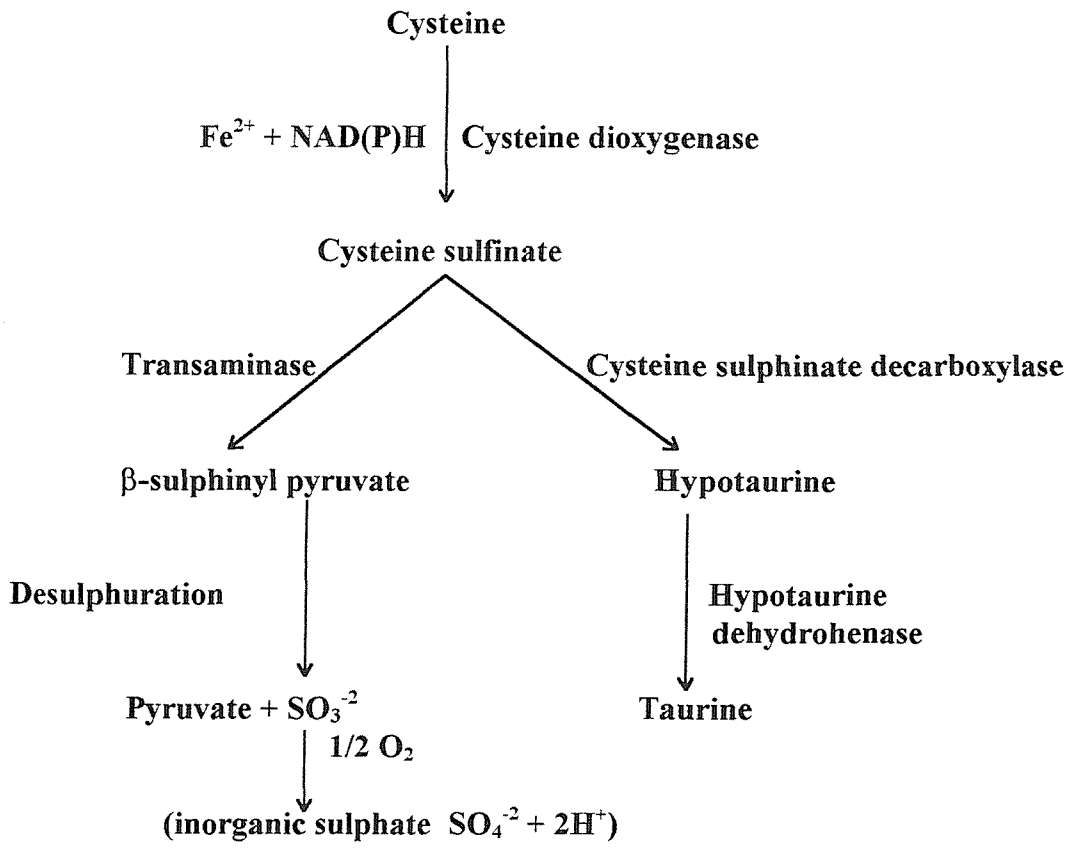
The metabolism of cysteine can be divided into two main routes:

1) The cysteine-conserving routes: used to synthesis GSH, and to make proteins.

2) The cysteine-catabolising routes: in which cysteine is broken-down to taurine and inorganic sulphate.

There is much evidence, which suggests that the catabolism of cysteine occurs predominately via the oxidation of cysteine to cysteine sulfinate in a reaction catalysed by cysteine dioxygenase (Yamaguchi *et al.* 1973; Stipanuk, 1979). Thereafter, cysteine sulfinate can be catabolised via the taurine pathway, or via the pyruvate pathway (Figure 1-15).

Figure 1-15 Cysteine degradation



In the taurine pathway, cysteine sulfinate is decarboxylated to form hypotaurine, in a reaction catalysed by cysteine sulfinate decarboxylase (Jacobsen *et al.* 1964). Finally, hypotaurine dehydrogenase converts hypotaurine to taurine (Reviewed in Horton *et al.* 1992). In the pyruvate pathway, cysteine sulfinate loses its amino

group by transamination to form β-sulfinyl pyruvate which is then decomposed to pyruvate and sulphite. Sulphite is then oxidised by sulphite oxidase to form sulphate (Macleod *et al.* 1961). Daniels & Stipanuk (1982) and Bagley & Stipanuk (1995) have observed that the activity of cysteine dioxygenase in rat hepatocytes was not significantly different between rats fed a diet deficient in sulphur amino acids and rats fed adequate amounts of it, but it was 3-17 times greater when sulphur amino acids were given in excess. This observation implies that animals are unable to conserve cysteine by a reduction in catabolism but that the pathway was responsive to potentially toxic quantities of the amino acid. It is therefore apparent that changes in glutathione or protein synthesis are important mechanisms in the conservation of cysteine in the body. In an in vitro study, Stipanuk *et al.* (1992) have demonstrated that cysteine metabolism to its major metabolites (GSH, taurine, sulphate) in rat hepatocytes was greatly affected by cysteine concentration. As cysteine concentration was increased, the formation of glutathione, sulphate and taurine were increased on a molar basis. However, the percentage of total cysteine metabolism that resulted in glutathione, taurine, and sulphate production in incubations with 0.05 mmol/l [^{35}S] cysteine + bathocuproine disulphonate (to prevent the auto-oxidation of cysteine) were 41%, 15%, and 44% respectively, whereas the percentage of cysteine metabolism to GSH, taurine, and sulphate in incubation with 1.0 mmol/l [^{35}S] cysteine were 19%, 34%, and 47% respectively. The main conclusions that were obtained from this study were that under conditions of low cysteine availability, glutathione synthesis was favoured, under conditions of high cysteine availability, taurine production was favoured, and that the percentage of cysteine converted to sulphate was unaffected by cysteine availability.

Observation of the kinetics of enzymes on the main pathways for cysteine metabolism (protein, glutathione, and sulphate and taurine) provides an interesting insight. The reported K_m of L-cysteinyl-tRNA synthetase, γ-glutamylcysteine synthetase, and for cysteine dioxygenase were 0.003mmol/L (Waterson *et al.* 1974), 0.35 mmol/L (Richman & Meister, 1975), and 0.45 mmol/L (Yamaguchi *et al.* 1978) respectively. This may indicate that even under low concentration of cysteine, L-

cysteinyl-tRNA synthetase can achieve maximum catalytic efficiency, and the priority will be for protein synthesis. Conversely, the formation of GSH, and sulphate and taurine will increase in proportion with cysteine availability in the cell. But, as already mentioned, relatively more cysteine will be converted to taurine than GSH as the intracellular concentration of cysteine rises. Thus factors, other than the kinetic properties of enzymes, determine the flow of metabolites along the various pathways of sulphur amino acid metabolism.

Some evidence suggests that cysteine can be catabolised by cysteine sulphinate independent pathway which leads to the formation of reduced sulphur and pyruvate. There are two cysteine sulphinate independent pathways that occur in mammalian tissues. These are, either by transamination of cysteine to produce 3-mercaptopyruvate followed by sulphur transferation, or by the cleavage of cysteine by cystathionase forming pyruvate, NH_3, and thiocysteine. Rhodanese catalyses the transfer of sulphur from thiocysteine to many acceptors such as cyanide (reviewed in Devlin, 1992).

1.8.4 Sulphur amino acids and the growth process

In the biosynthesis of proteins, cysteine is incorporated as a part of peptide chains via cysteine-tRNA. The sulfhydryl group (SH), the side chain of cysteine, is very important in stabilising the structure of proteins by forming a covalent disulphide linkage (Greenberg, 1975). The disulphide groups participate in the formation and stabilisation of the secondary, tertiary and quaternary structure of proteins, either by joining different parts of a single polypeptide chain to form loops within the chain or by joining different peptide chains. An example of proteins with intrachain S-S bound are albumin, and A chain of insulin, and an example of proteins with interchain S-S bound are between A and B chains of insulin, and between the light and heavy chains in immunoglobulin (reviewed in Torchinsky, 1981).

Although cysteine can be synthesised from methionine, there are indications that there is a requirement for the amino acid in the diet of growing rats. When methionine was provided at the minimum requirement needed to support growth (0.17%), the addition of cysteine to such a diet improves the growth performance of growing rats (Sowers *et al.* 1972 & Stockland *et al.* 1973). Cho *et al.* (1984) have observed that the mean daily weight gain of rats fed diets providing adequate or excess amounts of sulphur amino acids, in the form of methionine or cystine, was significantly higher than rats fed diet providing only the absolute minimum methionine requirement of growing rats (0.17%) recommended by Sowers *et al.* (1972) and Stockland *et al.* (1973). Alhamdan & Grimble (1997) have shown that the average daily weight gain of rats fed a low-protein diet (80 g protein/kg diet), supplemented with 1.46 g cysteine/kg diet, was higher than animals fed the low-protein diet, but without cysteine supplementation. The total amount of sulphur amino acids present in the former diet was 32 mmol/kg diet, and in the latter diet it was 20 mmol/kg diet.

1.8.5 The role of sulphur amino acids in modulating the metabolic response to an inflammatory stimulus

Since the production of many compounds containing sulphur amino acids are increased during inflammation, such as glutathione, and a wide range of acute phase proteins, the dietary supply of cysteine may need to be increased to satisfy the need for these compounds, particularly glutathione and metallothionein which are rich in the amino acid (Grimble, 1992). Consequently, by providing the required substrates in adequate amounts for synthesising these compounds, the body can improve its ability to control the effects of oxidant molecules and the production of cytokines in response to inflammatory stimuli.

The availability of sulphur amino acids has a strong influence on the glutathione content in liver, lung, spleen, thymus, muscle, and heart, both in the presence and absence of inflammatory stimuli (Cho *et al.* 1984; Bauman *et al.* 1988

a & b; Hunter & Grimble, 1994). After giving endotoxin to young growing rats, previous experiments carried out in our laboratory, showed the ability of either methionine or cysteine supplementation to enhance glutathione concentration in liver and lung during an inflammatory response in animals fed a low-protein diet (Hunter & Grimble 1994). Bauman *et al.* (1988b) have shown that rats fed on 75 g protein/kg diet, had lower hepatic GSH than rats fed on 150 g protein/kg diet, using casein as a source of protein. However, when the 75 g protein/kg diet was supplemented with L-2-oxothiazolidine-4-carboxylate (OTC), a stable derivative of cysteine, to be isosulphurous with the 150 g protein/kg diet, hepatic GSH concentration in animals fed the 75 g protein + OTC/kg diet was not different from that in rats fed the 150 g protein/kg diet. Paaw & Davis (1990) have observed that taurine, which is the major end product of cysteine, is decreased in injured patients. This may indicate the diversion of cysteine into cysteine rich compounds, such as glutathione and metallothionein, rather than being metabolised in the taurine synthetic pathway. Askanazi *et al.* (1980) have shown that glycine, the precursor of serine, and serine, the provider of the carbon skeleton of cysteine in the first step of the transsulphuration pathway, are decreased in the plasma of injured and septic patients. These patients have also experienced a reduction in the plasma taurine. These data may indicate an increase flux of substrate through the transulphuration pathway, toward cysteine biosynthesis, as indicated by the decrease in plasma glycine and serine levels. Furthermore, the cysteine-conserving routes, used to synthesis GSH or protein may be favoured over the cysteine-catabolising routes, as indicated by the decreased in plasma taurine level. The increased synthesis of GSH and protein, which might be expected in such patients, may also account for the decrease in plasma glycine. A reduction in the ratio of urinary nitrogen excretion to sulphate excretion was found in patients undergoes an abdominal aortic aneurysm repair operation, indicating a preferential retention of sulphur amino acids (Grimble, 1994a). In burn patients, with burns covering more than 30% of the body surface, the excretion of urinary nitrogen was enhanced to a greater extent than the excretion of total sulphur (Larsson *et al.* 1982). L-S^{35}-labelled cysteine incorporated into

anionic fractions of tissues, containing sulphate, was lower in liver, spleen, kidney, and heart of infected rats compared to pair-fed rats (Malmezat *et al.* 1998).

As indicated earlier (section 1.7), cysteine is also important to optimise immune function. Gmunder *et al.* (1990) have reported that macrophages utilise cystine and generate thiol compounds, mainly as cysteine. The cysteine released was used by lymphocytes and T cell clones, which have a strong transport activity for cysteine. Consequently, the intracellular glutathione level in the lymphocytes was increased. Gmunder *et al.* (1991) have also reported that T cells required cysteine which comes primarily from extracellular cysteine. Furthermore, this study has shown that when human T lymphocytes were mitogenically stimulated, the transport activity of cysteine was markedly increased, and the rate of DNA synthesis in these lymphocytes was found to be influenced by variations in the extracellular concentration of cysteine.

2. Aim of studies

Dietary recommendations for amino acids are designed to meet the requirements for growth, pregnancy, and day to day stresses of life in healthy populations. However, stimulation of the immune system in infected, traumatised patients may increase demands for specific amino acids over and above the amounts in these recommendations. Sulphur amino acids, tryptophan, phenylalanine, tyrosine, and arginine are among this specific group of amino acids.

As indicated earlier, many studies suggest the possible role of the sulphur amino acids in modulating the metabolic response to an inflammatory stimulus, and there is evidence in the literature which suggests an increased demand for sulphur amino acids during an inflammatory response (Askanazi *et al.* 1980; Grimble 1992; Gmunder *et al.* 1992; Hunter & Grimble 1994; Paaw & Davis, 1990). Furthermore, many studies showed the importance of sulphur amino acids in supporting the growth process (Sowers *et al.* 1972; Stockland *et al.* 1973; Cho *et al.* 1984; Alhamdan & Grimble, 1997). Reeds *et al.* (1994) have suggest that the profile of amino acids released from skeletal muscle protein during the acute phase response is relatively deficient in the aromatic amino acids (phenylalanine, tryptophan, and tyrosine) in relation to the amino acid composition of the acute phase proteins. There appears, therefore, to be a mismatch between skeletal muscle protein amino acid composition and the amino acid requirement for the acute phase protein response. The consequence of this situation is that when muscle protein is catabolised in amount that is sufficient for the acute phase response, some amino acids will be released in quantities that are surplus to these requirements. The surplus amino acids are catabolised contributing to the negative nitrogen balance. Thus, there may be specific amino acid requirements for the response, which if met, will thereby improve nitrogen balance.

While the true extent of the mismatch between amino acid outflow from peripheral tissues and the requirement for an acute phase response is ill defined, if

such a phenomenon occurs, dietary intervention by adding sulphur and aromatic amino acids to the diet could produce major therapeutic benefits.

Neither Reeds *et al.* (1994), nor our preliminary conclusions, identify a shortfall in amino acids other than sulphur and aromatic amino acids, but there is an increasing body of evidence that other amino acids may exert a 'beneficial effect' following trauma and infection. Arginine is among these beneficial amino acids. Arginine could have a modulatory effect on nitrogen metabolism, as a precursor of urea, and on immune functions, as a precursor of ($NO^{\bullet}$). However, arginine supplementation has been shown to have immunoenhancing effects in normal subjects, postoperative patients, patients on intensive care and HIV positive patients (Kirk & Barbul, 1990). In rats addition of arginine and glycine to a 200 g/kg casein diet reduced the extent of the negative N balance following bone fracture (Sitren & Fisher, 1977).

The objective of the research reported in this thesis is to identify the relative importance of sulphur amino acids, tryptophan, and arginine, on the response to an inflammatory agent and during growth, using a rat model. The thesis describes a series of inter-related studies. In the first study, differing degrees of sulphur and other essential amino acid deficiency were created by feeding animals diets of differing degrees of protein deficiency, using casein as a sole protein source. In the second, third and fourth studies, diets contain supplements of L-methionine, L-tryptophan and L-arginine, in various combinations. The additions were in amounts which bring the total amount of each supplemental amino acid to the concentration found in a diet containing 180 g/kg diet with an additional 3 g L-methionine/kg diet, a diet which is considered adequate for young growing rats. In all studies, rats were divided into three treatment groups, after feeding the diets *ad libitum* for seven days. One-third of each group was injected with endotoxin (lipopolysaccharide from Escherichia coli) and allowed free access to food for a further 24 hours. One-third of each group was pair-fed with an amount of diet similar to that consumed by their littermates injected with the endotoxin. The final one-third of each group continued to be fed *ad libitum*. To reach the objective, the specific aims of the studies are:

1) To investigate the influence of dietary sulphur amino acid sufficiency on growth.

2) To investigate the influence of dietary sulphur amino acid, tryptophan, and arginine sufficiency on glutathione concentration in liver, lung, spleen, and thymus in rats undergoing an inflammatory response.

3) To investigate the effect of dietary sulphur amino acid, tryptophan, and arginine sufficiency on liver and lung protein content, and on acute phase proteins during inflammation.

4) To investigate the influence of dietary sulphur amino acid, tryptophan, and arginine sufficiency on the excretion of end products of amino acid catabolism (inorganic sulphate, urea-N and ammonia-N) during growth and in rats undergoing an inflammatory response.

5) To investigate how the dietary variables influence the partitioning of sulphur amino acids into their major end products (hepatic glutathione and protein, inorganic sulphate excretion and nitrogenous excretion products) during growth and inflammation, and the impact, which changes in partitioning, has on neutrophils present in the lung.

CHAPTER TWO

2. Materials and Methods

2.1 Materials

All reagents were obtained from **Sigma Chemical Company**, Poole, Dorset, England. Exceptions were:

BDH Limited, Poole, Dorset, England

Metaphosphoric acid (Glacial)

di-sodium hydrogen orthophosphate

Fisons Scientific Equipment, Bishop Moadow Road, Loughborough, England

Sodium sulphate

Ammonium sulphate

Cardiotech Services, Louisville, ky, USA

α-1 acid glycoprotein (standard)

Agar plates containing the antiserum to rat α-1 acid glycoprotein

Special Diets Services, Witham, Essex, England

Casein

Cellulose (Solkafloc)

Maize starch

Mineral Mix (AIN-76 mix)

Vitamin Mix (AIN-76 mix)

Mazola

Maize oil

The Silver Spoon Company, British Sugar plc, Peterborough, England

Sucrose

2.2 Methods

2.2.1 Protein concentration

The chromogenic reagent, Bicinchoninic acid (BCA), was used to determine the protein concentration in tissues (Smith *et al.* 1985). This method based on the principle that protein has the ability to reduce copper (11) to copper (1) in an alkaline environment (Biuret reaction), in a concentration dependent manner. The BCA is capable of forming an intense purple colour complex with Cu^{+1}, the intense purple colour produced is increased in a proportional fashion to protein concentration.

-Biuret reaction

a) Protein $+Cu^{+2} \longrightarrow Cu^{+1}$

b) Cu^{+1} + BCA $\longrightarrow$ BCA-Cu^{+1} complex.

Reagents

- BCA reagent consists of an aqueous solution of:

1% BCA-Na_2, 2% $Na_2CO_3.H_2O$, 0.16% Na_2-tartate, 0.4% NaOH and 0.95% $NaHCO_3$

- 4% (w/v) copper sulphate solution

Working colour protein determinant reagent

Protein determinant reagent was prepared fresh by mixing 1 volume of the 4% copper sulphate solution to 50 volume of bicinchoninic acid solution.

Standard: standard was prepared from bovine serum albumin over the concentration range 0-1.0 mg/ml.

Procedure

Approximately 400 mg of liver (200 mg for lung, spleen and thymus) weighed accurately was homogenised in 4 ml of distilled water (2 ml for lung, spleen and thymus). Then, 20 µl aliquot of the homogenised sample was diluted 1/20 for liver and 1/10 for lung, spleen, and thymus. A 10 µl aliquot of sample (standard

and blank) was added to a microelisa plate in duplicate. A 200 µl of the protein determinant reagent was added to each well containing sample (standard and blank), and the plate was incubated at 37 °C for 30 minutes. Thereafter, the plate was cooled at room temperature, and the absorbance was read in a microelisa plate reader (EL 340, Bio Tek Instruments) at 562 nm.

$$\text{protein concentration (mg/g tissue)} = \frac{\text{mg/ml} \times \text{dilution factor}}{\text{Tissue sample weight (g)}}$$

2.2.2 Glutathione assay

The principle of this assay is based on the oxidation of the reduced form of glutathione by the aromatic disulphide compound, 5,5,- dithiobis-2-nitrobenzoic acid (DTNB), to form GSSG and the aromatic thiol, 5,thio-2-nitrobenzoic acid (MNB) (Beutler *et al.* 1963):

GSH + DTNB $\longrightarrow$ GSSG + MNB.

The yellow colour formed due to the reduction of DTNB was measured at 412 nm, and it is proportional to the amount of glutathione present in the sample.

Reagents

- Precipitation solution

1.67 g glacial metaphosphoric acid.

0.2 g disodium ethylenediamine tetraacetic acid (EDTA).

30 g sodium chloride.

Made up to 100 ml with distilled water. This solution is stable for approximately 3 weeks at 4°C.

- Phosphate solution

0.3 M Na_2HPO_4. This solution stores indefinitely unless mould is formed. If crystals develop during storage at 4°C, it was dissolved by heating at 37°C.

- DTNB reagent

40 mg DTNB

1 g sodium citrate

Made up to 100ml with D.water

Standard: Glutathione standards were made over the concentration range 0.0-0.1 mg/ml by diluted with the phosphate solution.

Procedure

A weighed portion of a fresh liver (0.08-0.12 g) or fresh lung, spleen and thymus (0.12-0.16 g) was homogenised in a tube containing 2.5 ml of ice cold precipitation solution. Further 2.5 ml of precipitation solution was added to the homogenised tissue, allowed to stand on ice for 5 minutes, then the solution was mixed and filtered. One ml of the filtered sample was added to 4.0 ml of phosphate solution, followed by 1.0 ml of DTNB reagent. The solution was vortex mixed and the absorbance of the (blank, sample, and standard) was immediately measured at 412 nm using a standard spectrophotometer.

Tissue glutathione concentration was calculated from:

$$\text{GSH (}\mu\text{mol/g tissue)} = \frac{\text{tissue GSH (mg/ml)} \times 5 \times 1000}{\text{tissue sample weight (g)} \times 307}$$

2.2.3 Sample preparation for glutathione enzyme assays

Homogenising buffer (pH 7.4) prepared by mixing 30 mM potassium chloride with 10 mM dipotassium phosphate was used.

Procedure for preparing the samples to determine glutathione enzyme activities

Approximately 400 mg of liver (lung, spleen and thymus) was homogenised to disrupt the cell membrane, in the above homogenising buffer using an ultraturax, to give a final concentration of exactly 100 mg tissue/ml. The homogenate was

sonicated using MSE Soniprep 150 sonicator for 1 min on a cycle of 6 × 10s with 5s intervals, amplitude 16, to disrupt the intracellular membranes. Samples were then aliquoted into micro-centrifuge tubes and centrifuged for 5 minutes. The supernatant was then frozen at -70°C for further analyses of glutathione enzyme activities.

Notes:

1) All samples were kept on ice during homogenisation and sonication.

2) Protein concentration was also determined in the supernatant of these samples using the bicinchoninic solution to express the samples as unit of enzyme activity/ mg or g protein.

2.2.4 Glutathione Peroxidase

This assay is based on the ability of glutathione peroxidase to oxidise glutathione in the presence of a peroxide (Wendel, 1981).

Glutathione peroxidase catalysed the oxidation of the reduced form of glutathione (GSH) to the oxidised form (GSSG) in the presence of a hydroperoxide. GSSG is immediately converted to GSH by the action of glutathione reductase. This action of glutathione reductase leads to the oxidation of NADPH to $NADP^+$. The decrease in the absorbance of NADPH was measured at 340 nm.

Reagents

- Tris-HCl/ EDTA (pH 8.0)

0.1 M Tris-HCl buffer

5.0 mM EDTA

- t-Butyl hydroperoxide diluted 1-10000 with distilled water.

- Substrate (Glutathione peroxidase reagent)

20 mg NADPH

61.4 mg glutathione

100 unit glutathione reductase

made up to 100 ml with tris-HCl/EDTA

Procedure

840 µl of glutathione peroxidase reagent was added to 80 µl of sample (blank). To this solution, 80 µl of t-butyl hydroperoxide was added and immediately the decline in the absorbance of NADPH was measured over 1 min at 340 nm.

The activity of glutathione peroxidase was calculated as U/ml = (sample-blank) × 2.01

2.2.5 Glutathione reductase

The principle of this technique, used to determine glutathione reductase in tissue, based on the oxidation of NADPH to $NADP^+$ by glutathione reductase in the presence of oxidised form of glutathione (GSSG) (Carlberg & Mannervik, 1975).

GSSG reductase reagent

To 0.10 M sodium phosphate buffer (pH 7.6), the following reagents were added:

- 1.0 mM GSSG
- 0.1 mM NADPH
- 0.5 mM EDTA

Procedure

30 µl of sample (blank) was placed in a microelisa plate in triplicate, this was followed by the addition of 200 µl GSSG reductase reagent. The decrease in the absorbance of NADPH was followed for 1 minutes at 30°C. The absorbance was read in a microelisa plate reader (EL 340, Bio Tek Instruments) at 340 nm.

GSSG reductase U/ml= (sample-blank) × 1.233

2.2.6 Method for collecting urine from rats

Metabolic cages were used to collect urine. Urine was collected using 30 ml plastic measuring cylinder attached to a funnel. At the end of each collection period (24 hour), from 9-10 a.m. to 9-10 a.m. next day, the inner surface of the cages was

washed down carefully with distilled water to ensure a complete collection of urine. Access to the food pot in the metabolic cages is by a tunnel, so the rat can not easily pass urine into it. A few drops of HCl were added to each plastic measuring cylinder to preserve urine.

2.2.7 Inorganic sulphate excretion in urine

The method is based on the turbidimetry of sulphate as barium sulphate in the presence of a very small amount of preformed barium sulphate, and with polyethylene glycol (PEG) as a stabilising agent (Lundquist *et al.* 1980):

Na_2SO_4 (urine-SO_4) + BaCl $\longrightarrow$ HCl + $BaSO_4$

The presence of preformed barium sulphate in the assay is necessary for the sulphate in the sample to be precipitated more reproducibly (Lundquist *et al.* 1980).

Reagents used in this assay

- Barium-PEG reagent

150.0 g PEG 6000

9.77 g Barium chloride dihydrate

Made up to 1 L with deionised water.

A 0.2 ml of 0.05 M Na_2SO_4 was added to 100 ml of the Barium-PEG reagent with efficient magnetic stirring.

Standard: Sodium sulphate was used over the concentration range 0.0-50 µmol/ml.

Procedure

Deionised water (3.0 ml) was added to 0.1 ml aliquot of urine (standard, blank). Then, 1.0 ml of 0.5 M HCl was added to the diluted urine (standard, blank). This was followed by 1ml of the Barium-PEG reagent, vortex mix, and left to stand for 5-10 minutes, before the absorbance of the sample (standard, blank) was determined on a standard spectrophotometer at 600 nm. The inorganic sulphate (SO_4) concentration as µmol/ml was obtained from the standard curve.

Excretion of SO_4 (µmol/24hours)= [µmol/ml × volume of urine (24 hours)].

Before starting treatments: urine was collected on day 7 and day 8, which represents the excretion of SO_4 for the last two days, before starting treatments. The result's mean was taken, and expressed as µmol SO_4/day.

After starting treatments: for the LPS treated-nimals, urine was collected on day 9, which represents the excretion of SO_4 on day 8, a period of 24 hours after endotoxin treatment. For the animals fed *ad libitum* and for pair-fed animals, urine was collected on day 10, which represents the excretion of SO_4 on day 9. The result was expressed as µmol SO_4/day.

2.2.8 Ammonia-N determination in urine using Berthelot reaction

Ammonia-N was measured using the phenate-hypochlorite method, Berthelot reaction, (Kaplan, 1965). Simply, ammonia-N reacts with phenol and alkaline hypochlorite to form a blue chromogen-indophenol, using sodium nitroprusside as a catalyst. The intensity of the blue colour formed increases in proportion to the amount of ammonia-N present in urine. The absorbance of the blank, standard and samples was measured at 560 nm using a standard spectrophotometer.

Reagents used to measure ammonia-N

- Alkaline hypochlorite

Stock: 25 g sodium hydroxide pellets in 800 ml water and 40 ml "Chlorax" bleach (5% NaOCl) made up to 1 litre. This reagent was stored in a dark bottle (stable for two months).

Working solution: 100 ml of stock added to 400 ml distilled water.

-Phenol nitroprusside solution:

Stock: 50 g phenol (analar) in 500 ml distilled water and 250 mg sodium nitroprusside in 100 ml distilled water dissolved separately and made up to 1 litre.

Working solution: 100 ml stock added to 300 ml distilled water.

Ammonia-N Standard

500 mg NH_4/100 ml. This was prepared by drying ammonium sulphate $(NH_4)_2SO_4$ in a glass beaker at 100°C. for 12 hours, then 2.36 g of the dried $(NH_4)_2SO_4$ was made up to 100 ml with distilled water.

-Working standard:

0	Distilled water (D.water)	0.0 µg NH_3-N/10µl
1	1 ml $(NH_4)_2SO_4$ + 49ml D. water	1.0 µg NH_3-N/10µl
2	2 ml $(NH_4)_2SO_4$ + 48ml D.water	2.0 µg NH_3-N/10µl
3	3 ml $(NH_4)_2SO_4$ + 47ml D.water	3.0 µg NH_3-N/10µl
4	4 ml $(NH_4)_2SO_4$ + 46ml D.water	4.0 µg NH_3-N/10µl
5	5 ml $(NH_4)_2SO_4$ + 45ml D.water	5.0 µg NH_3-N/10µl

Procedure

10 µl of blank, standard and urine sample was pipetted at the bottom of a test tube. To each tube, 4 ml of working phenol nitroprusside solution was added and mix, followed by the addition of 5 ml working alkaline hypochlorite solution and mix. The tubes, then, were incubated at 37°C for 20 minutes using a water bath. The absorbance of the blank, standard, and sample were read at 560 nm using a standard spectrophotometer.

$$\text{mg } NH_3\text{-N} = \frac{(\mu g\ NH_3\text{-N} \times 1000)}{10} /1000 \times \text{total volume of urine (ml/24 hours).}$$

Before starting treatments: urine was collected on day 7 and day 8, which represents the excretion of NH_3-N for the last two days, before starting treatments. The result's mean was taken, and expressed as µmol NH_3-N/day or mg NH_3-N when correlated with nitrogen intake.

After starting treatments: for the LPS treated animals, urine was collected on day 9, which represents the excretion of NH_3-N on day 8, a period of 24 hours after

endotoxin treatment. For the animals fed *ad libitum* and for pair-fed animals, urine was collected on day 10, which represents the excretion of NH_3-N on day 9. The result was expressed as μmol NH_3-N/day or mg NH_3-N when correlated with nitrogen intake.

2.2.9 Urea-N determination in urine using Berthelot reaction

Urea-N was measured by converting it to ammonia using the enzyme urease. Ammonia-N can be measured using the phenate-hypochlorite method, Berthelot reaction, (Kaplan, 1965). Simply, after converting urea to ammonia, ammonia-N reacts with phenol and alkaline hypochlorite to form a blue chromogen-indophenol, using sodium nitroprusside as a catalyst. The intensity of the blue colour formed increases in proportion to the amount of ammonia and indirectly to the amount of urea-N present in urine. The absorbance of the blank, standard and samples was measured at 560 nm using a standard spectrophotometer.

Reagents used to hydrolysed Urea

-EDTA

27 mM Na_2-EDTA adjust to pH 6.5 with 30% NaOH

-Urease solution (30 units/ml)

50 mg jack bean urease type III (30000 units/g) was dissolved in 25 ml distilled water and 25 ml glycerol was added. store at 4°C.

-Working solution

1ml of stock urease diluted in 100 ml EDTA buffer. Stored in Plastic bottle at 4°C. This solution is stable for one month.

Reagents used to measure urea-N

- Alkaline hypochlorite

Stock: 25 g sodium hydroxide pellets in 800 ml water and 40 ml "Chlorax" bleach (5%NaOCl) made up to 1 litre. This reagent was stored in a dark bottle (stable for two months).

Working solution: 100 ml of stock added to 400 ml distilled water.

-Phenol nitroprusside solution

Stock: 50 g phenol (analar) in 500 ml distilled water and 250 mg sodium nitroprusside in 100 ml distilled water dissolved separately and made up to 1 litre.

Working solution: 100 ml stock added to 300 ml distilled water.

Urea-N standard

-Stock: 500 mg urea-N/100 ml. 1.0717 g urea and 100 mg sodium azide (as a preservative) made up to 100 ml.

-Working standard:

D.water (0 µg urea-N/25µl).

1 ml stock + 49 ml D.water (2.5 µg urea-N/25µl).

2 ml stock + 48 ml D.water (5 µg urea-N/25µl).

3 ml stock + 47 ml D.water (7.5 µg urea-N/25µl).

4 ml stock + 46 ml D.water (1 µg urea-N/25µl).

Procedure

Urine was diluted 1:20 (50 µl of urine in 950 µl D.water), then 25 µl of blank, diluted sample, and standard was pipetted to the bottom of a test tube. To each tube 1ml of working urease was added and incubated for 20 minutes at 37°C. To these tubes, 4.0 ml of working phenol nitroprusside solution was added and mix, followed by the addition of 5.0 ml working alkaline hypochlorite solution and mix. The tubes, then, were incubated at 37°C. for 20 minutes using a water bath. The absorbance of the blank, standard, and sample was read at 560 nm using a standard spectrophotometer.

1) mg urea-N + NH_3-N= $\frac{\text{µg Urea-N + NH3-N}}{25} \times 20 \times$ total volume of urine (ml/ /24 hours)

2) mg urea-N/day = (Urea-N + NH_3-N) – NH_3-N

Before starting treatments: urine was collected on day 7 and day 8, which represents the excretion of urea-N for the last two days, before starting treatments. The result's mean was taken, and expressed as μmol urea-N/day or mg urea-N when correlated with nitrogen intake.

After starting treatments: for the LPS-treated animals, urine was collected on day 9, which represents the excretion of urea-N on day 8, a period of 24 hours after endotoxin treatment. For the animals fed *ad libitum* and for pair fed animals, urine was collected on day 10, which represents the excretion of urea-N on day 9. The result was expressed as μmol urea-N /day or mg urea-N when correlated with nitrogen intake.

2.2.10 α-1 acid glycoprotein

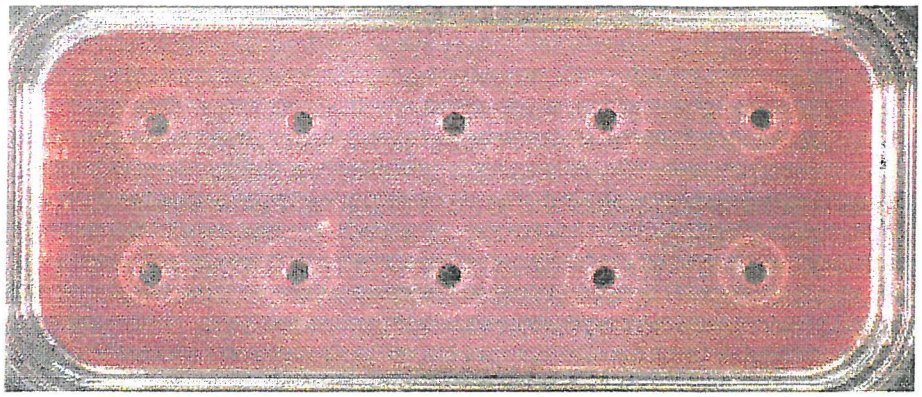
Serum α-1 acid glycoprotein was measured by single radial immunodiffusion assay (SRID). One of the best reviews of this assay can be found in the paper of Fahey & Mckelvery (1965). The principle of this assay is that serum diffuses radially from a small antigen well cut in an agarose plate containing specific antiserum to α-1 acid glycoprotein. A specific precipitin reaction occurs between rat α-1 acid glycoprotein and the antiserum incorporated in the gel. A visible precipitin ring is formed. The area within this ring is directly proportional to the concentration of α-1 glycoprotein in the plasma. The measurement of the ring's diameter allows calculation of α-1 acid glycoprotein as compared to the standard.

Procedure

An Agar plate containing the antiserum to rat α-1 acid glycoprotein was purchase from Cardiotech Services (Louisville, USA). Each well was filled with 5 μl of serum sample using capillary pipette, and the plate cover was securely replaced. The plates were then incubated in a humidified-plastic box at room temperature for 48 hours before reading the test results. The precipitate ring diameters were measured using electronic RID plate reader, and the α-1 acid glycoprotein standard

was calculated using linear regression. Figure 2-1 shows a scanned photo of an Agar plate, after 48 hours of filling each well with 5 µl of serum sample.

Figure 2-1 An Agar plate containing the antiserum to rat α-1 acid glycoprotein with a precipitate ring formed around each well filled with one of our serum samples



2.2.11 Albumin concentration

The bromocresol green dye method, using glycine-HCl as a buffer, was used to determine the concentration of the albumin in the serum (McPherson & Everad, 1972). Albumin binds with bromocresol green reagent (pH 3.8) forming an intense green color. The intensity of the colour is directly proportional to albumin concentration.

Reagents

- 1 M Glycine
- 1 M HCl
- 0.02 M Bromocresol green (B.C.G.)-alkaline-dissolved in ethanol.
- 30 % Brij 35

Working colour reagent (bromocresol green reagent):

To 500 ml of water, 94.5 ml glycine, 5.5 ml HCl, 4 ml Brij 30% and 3.0 ml of 0.02 M B.C.G. were added. Make up to 1 L with d. water, and adjusted to pH 3.8.

Standard: Bovine serum albumin was used as a standard over the concentration 0-50 mg/ml.

Procedure

To 20 µl of serum (standard, blank) 5.0 ml of bromocresol green reagent was added. The solution was mix, and read immediatly againest a reagent blank in a standard spectrophotometer at 635 nm.

2.2.12 Neutrophilic cells in lung

The number of neutrophils was determined using a light microscope. This was done by counting the number of neutrophils in the total section.

Reagents

Neutral buffered formalin (pH 7.4) was made by mixing the following compounds:

3.5 g sodium dihydrogen orthophosphate

6.5 g disodium hydrogen orthophosphate

100 ml 37% formalin

Made up to 1000 ml with distilled water.

Procedure of counting neutrophilic cells in lung

Small samples of lung tissues were dissected from rats, and then immediately preserved in neutral buffered formalin (NBF). Slides of these NBF-preserved samples were kindly prepared by MR. R. Lee (Department of Pathology-Southampton General Hospital). This was done by fixing and embedding the lung samples in paraffin wax, before cutting (3 µm sections), and staining with haematoxylin and eosin. Slides were then studied under light microscope.

At low-power field magnification (10 ×), Point counting was used to measure the surface area of lung. Lung section occupied by intersection point of the graticule was counted as one point surface area (see Figure 2-2 Illustration of counting the surface area of lung section). After the total lung area was determined, the total number of neutrophils seen in the total lung section was counted, using high-power magnification (100×oil-immersion objective). The number of neutrophils was

standardised by dividing the total number of neutrophils over the total number of the points-surface area occupied by lung section. The result was multiply by 100:

$$\text{Percentage of neutrophils in total lung section} = \frac{\text{Total number of neutrophils}}{\text{Total number of points}} \times 100$$

Figure 2-2 Illustration of counting the surface area of lung section

Figure 2-1 (a)
Intersection points in graticule

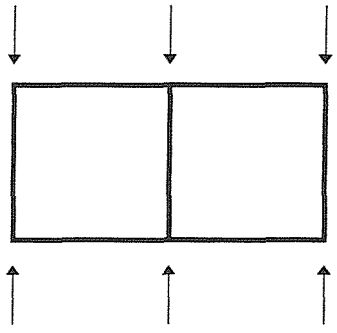


Figure 2-1 (b)

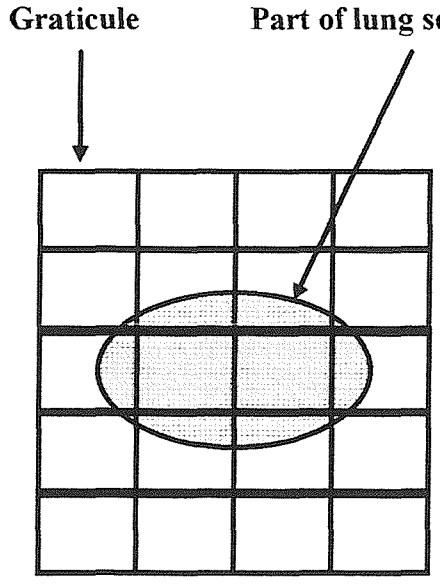


Figure 2-1 (c)

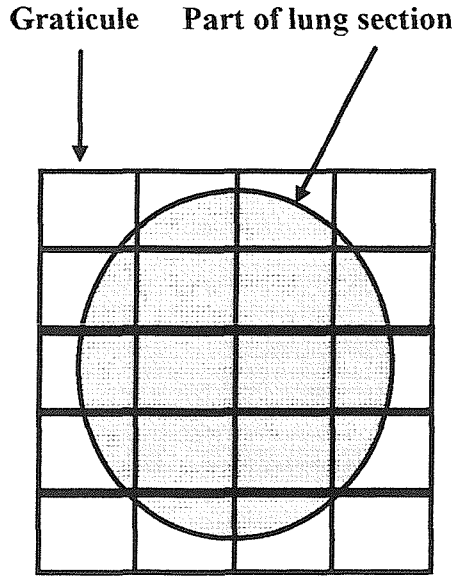


Figure 2-1 (b): lung area occupied by intersection points in the graticule is 6 (6 points of surface area).
Figure 2-1 (c): lung area occupied by intersection points in the graticule is 12 (12 points of surface area).

CHAPTER THREE

3. Coefficient of variation and Statistical analysis

3.1 Coefficient of variation

In order to assess the variation in results for a given method a series of validation experiments were performed where possible. Coefficient of variation was calculated, from the mean (M) and standard deviation (SD), to evaluate the variation in result.

$$\textbf{Coefficient of variation} = \frac{SD \times 100}{M}$$

Coefficient of variation was determined by measuring one sample 10 times on the same day (intra-assay), and by measuring one sample 10 times, where possible, in separate days (inter-assay).

Table 3.1 Coefficient of variation for protein assay

Protein (mg/g liver) Intra-assay		**Protein (mg/g liver)** Inter-assay	
Analysis 1	172.4	Day 1	144.4
Analysis 2	165.0	Day 2	164.6
Analysis 3	159.2	Day 3	155.0
Analysis 4	156.4	Day 4	141.0
Analysis 5	159.8	Day 5	159.0
Analysis 6	154.8	Day 6	151.0
Analysis 7	162.6	Day 7	139.0
Analysis 8	156.4	Day 8	135.0
Analysis 9	158.0	Day 9	136.0
Analysis 10	150.2	Day 10	142.2
3.8 %		6.9 %	

Table 3.2 Coefficient of variation for glutathione peroxidase

Glutathione peroxidase (U/g protein) Intra-assay		**Glutathione peroxidase (U/g protein) Inter-assay**	
Analysis 1	33.0	Day 1	28.9
Analysis 2	34.9	Day 2	32.6
Analysis 3	30.9	Day3	33.6
Analysis 4	30.7	Day 4	31.9
Analysis 5	31.6	Day 5	31.6
Analysis 6	32.3	Day 6	32.4
Analysis 7	30.7	Day 7	32.8
Analysis 8	32.1	Day 8	29.1
Analysis 9	31.6	Day 9	27.8
Analysis 10	31.1	Day 10	27.0
4.0 %		7.6 %	

Table 3.3 Coefficient of variation for inorganic sulphate excretion

Inorganic sulphate excretion (µmol/day) intra-assay		**Inorganic sulphate excretion (µmol/day) inter-assay**	
Analysis 1	157	Day 1	157
Analysis 2	153	Day 2	155
Analysis 3	161	Day 3	166
Analysis 4	155	Day 4	160
Analysis 5	159	Day 5	174
Analysis 6	162	Day 6	160
Analysis 7	161	Day 7	165
Analysis 8	155		
Analysis 9	158		
Analysis 10	159		
1.9 %		4.0 %	

Table 3.4 Coefficient of variation for ammonia-N excretion

Ammonia-N (mg/day) intra-assay		**Ammonia-N (mg/day) inter-assay**	
Analysis 1	12.0	Day 1	12.0
Analysis 2	11.3	Day 2	12.4
Analysis 3	12.2	Day3	11.2
Analysis 4	11.5	Day 4	12.5
Analysis 5	11.0	Day 5	11.1
Analysis 6	10.8	Day 6	12.8
Analysis 7	12.0	Day 7	12.1
Analysis 8	12.5	Day 8	10.1
Analysis 9	12.8	Day 9	11.0
Analysis 10	11.4	Day 10	12.0
5.6 %		7.1 %	

Table 3.5 Coefficient of variation for serum albumin concentration

Albumin (mg/ml) intra-assay		**Albumin (mg/ml) inter-assay**	
Analysis 1	33.5	Day 1	33.5
Analysis 2	31.5	Day 2	32.3
Analysis 3	32.0	Day3	34.0
Analysis 4	33.7	Day 4	29.5
Analysis 5	33.0	Day 5	33.0
Analysis 6	31.5	Day 6	35.0
Analysis 7	32.0	Day 7	33.7
Analysis 8	32.3	Day 8	32.5
Analysis 9	31.5	Day 9	33.2
Analysis 10	32.3	Day 10	31.5
2.5 %		4.6 %	

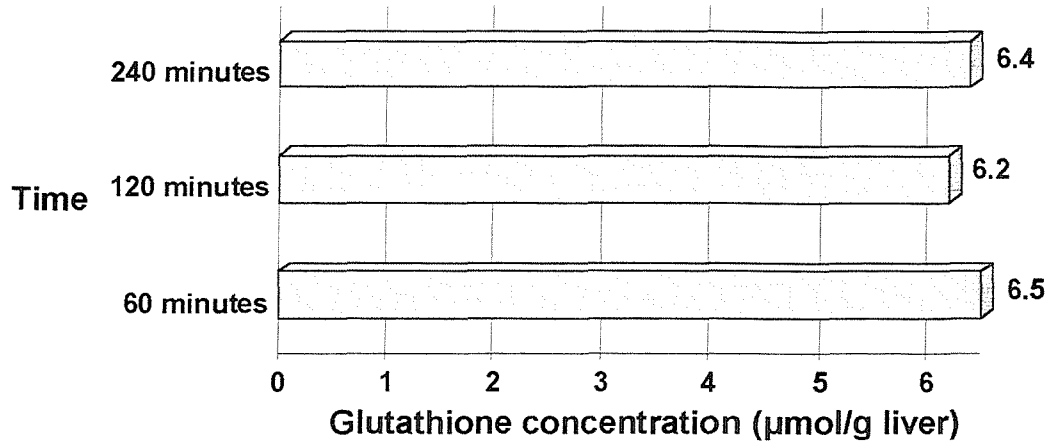
Table 3.6 Coefficient of variation for serum α-1 acid glycoprotein concentration

α-1 acid glycoprotein (µg/ml) intra-assay		α-1 acid glycoprotein (µg/ml) inter-assay	
Analysis 1	750	Day 1	750
Analysis 2	840	Day 2	820
Analysis 3	790	Day 3	860
Analysis 4	810	Day 4	820
Analysis 5	820	Day 5	730
Analysis 6	710	Day 6	700
Analysis 7	780	Day 7	790
Analysis 8	810	Day 8	840
Analysis 9	820	Day 9	790
Analysis 10	790	Day 10	710
4.8 %		7.1 %	

3.2 Time course stability of glutathione

Glutathione stability over four hours period was conducted, by repeating the assay of one filtered sample (see chapter 2, section 2.2.2 Glutathione assay), three times. First measurement was taken 60 minutes after dissecting the liver from rat (Figure 3-1).

Figure 3-1 The stability of glutathione measurement over a period of four hours.



3.3 Statistical analysis

Results are expressed as mean values ± standard error of the mean (SE). If the diet was the only variable, before dividing the animals into three treatment groups (LPS-treated animals, animals fed *ad libitum*, and pair-fed controls), the results were compared using one-way analysis of variance (one-way ANOVA). Differences in mean values with $p<0.05$ were considered significant. Where significant effects were found, differences between dietary groups were examined using unpaired students t-test with a significant level of $p<0.05$. If the diet and treatments were the two variables, after dividing the animals into three treatment groups (LPS-treated animals, animals fed *ad libitum*, and pair-fed controls), the results were compared using two-way analysis of variance (two-way ANOVA), Differences in mean values with $p<0.05$ were considered significant. Where significant effects were found, differences between treatment groups were examined using unpaired students t-test with a significant level of $p<0.05$.

CHAPTER FOUR

4. The effect of graded levels of dietary casein on growth and inflammatory responsiveness in rats

4.1 Introduction

The dietary intake, and availability of sulphur amino acids, have a strong influence on glutathione content of various organs such as liver, lung, spleen, thymus, muscle, and heart (Cho *et al.* 1984; Bauman *et al.* 1988a; Hunter & Grimble 1997), and on growth (Sowers *et al.* 1972, Stockland *et al.* 1973; Cho *et al.* 1984; Alhamdan & Grimble 1997).

Hum *et al.* (1992) have shown that in rats fed *ad libitum* graded amounts of dietary protein, hepatic glutathione concentration was increased as dietary protein content was increased from 0 g to 200 g protein/kg diet. Bauman *et al.* (1988a) have shown that hepatic glutathione concentration in rats fed low-protein diets (45 g and 75 g protein/kg diet) was significantly lower than in rats fed a normal-protein diet (150 g/kg diet). When the 75 g protein/kg diet was supplemented with cysteine, methionine, or DL-methionine hydroxy-analog, to be equivalent to the sulphur amino acid content of the 150 g protein/kg diet, hepatic glutathione concentration was increased to the level found in animals fed the 150 g protein/kg diet.

The relationship between dietary sulphur amino acid intake, glutathione, and protein metabolism during physiological stress is largely unknown. In the study described in this chapter, the effect of sulphur amino acid insufficiency on growth, hepatic and lung protein, α-1 acid glycoprotein and albumin in serum, number of neutrophils present in lung, and urinary end products of amino acid metabolism was examined in animals receiving endotoxin, fed *ad libitum*, or in pair-fed groups on whose diet was restricted to that of animals receiving endotoxin. In the study, glutathione concentrations in liver, lung, as well as spleen, and thymus, were examined in animals fed *ad libitum*, endotoxin-treated rats, and pair-fed controls. As

far as is known, no study has measured spleen and thymus glutathione concentration after endotoxin treatment in vivo. Furthermore, the enzymes involve in glutathione metabolism, glutathione peroxidase, and glutathione reductase, were also examined. The partitioning of sulphur amino acids into their main end products was measured indirectly by examining the ratios of products (urinary inorganic sulphate and nitrogenous excretion products, hepatic protein and glutathione). Finally, the number of neutrophils present in lung was counted.

4.2 Protein and sulphur amino acid content of the various diets

All diets were prepared by mixing dry ingredients in a mixer, this was followed by addition of maize oil. To these diets, water was added to make the diets into small biscuits that were dried in oven at 80^0C for up to 48-72 hours.

All groups were fed casein as the sole protein source in the diet (Table 4.1). Casein was chosen as a the protein source for two reasons:

a) It contains relatively low amounts of sulphur amino acids, when compared with other essential amino acids. Thus, by reducing the amount of dietary casein, sulphur amino acids become the most limiting amino acids (Table 4.1).

b) Using casein would allow us to make comparison with other studies (casein studies) conducted either in our laboratory or other laboratories (e.g. Hunter & Grimble, 1994; Bauman & Bray, 1988; Taylor *et al.* 1992; Bella & Stipanuk, 1995; Morand *et al.* 1997; Bagley & Stipanuk, 1995; Stiren & Fisher, 1977).

The casein powder contained 88% protein, and the amount of sulphur amino acids in casein, determined by HPLC, were 2.76 g methionine and 0.43 g cystine/100 g casein (Special Diets Services, 1995).

In order to achieve the aim of this study, four different groups of animals were used:

1) The first group received 180 g protein containing 45 mM sulphur amino acids/kg diet. This group is called the normal-protein (NP) group as sulphur amino acid content closely matched the requirements for young laboratory rats (Table 4.2).

2) The second group received 120 g protein containing 30 mM sulphur amino acids/kg diet. This group is called the moderate-protein (MP) group. This diet provided 73% of requirements for sulphur amino acids.

3) The third group received 80 g protein diet containing 20 mM sulphur amino acids/kg diet. This group is called the low-protein (LP) group. This diet provided 48% of requirements for sulphur amino acids.

4) The fourth group received 60 g protein containing 15 mM sulphur amino acids/kg diet. This group is called the very low-protein (VLP) group. This diet provided 36% of requirements for sulphur amino acids.

Table 4.1 Diets composition (g/kg)

Component	180 g protein/kg	120 g protein/kg	80 g protein/kg	60 g protein/kg
Casein	204	137	91	68
Cellulose	100	100	100	100
Sucrose	320	354	377	388
Maize starch	321	354	377	389
Maize oil	30	30	30	30
Vitamin mix	5	5	5	5
Mineral mix	20	20	20	20

Table 4.2 Amino acid requirements of growing rats, the amino acid composition of casein, and the amount of amino acids, expressed as g and as a percentage of the amino acid requirement, present in 100 g diet containing 18 g protein (NP), 12 g protein (MP), 8 g protein (LP) and 6 g protein (VLP), using casein as the sole source of protein. Deficient amino acid contents are shown in bold. [E]

amino acids	requirement g/100g diet*	amino acids composition / 100 g casein.♣	NP		MP		LP		VLP	
			g	%	g	%	g	%	g	%
Aspartic acid	0.40	6.03	1.23	308	0.83	206	0.55	137	0.41	102
Threonine	0.50	3.71	0.76	151	0.51	102	**0.34**	**68**	**0.25**	**50**
Glutamic acid	4.00	20.38	4.16	104	**2.79**	**70**	**1.85**	**46**	**1.38**	**35**
Proline	0.40	9.59	1.96	489	1.31	328	0.87	218	0.65	163
Valine	0.60	3.78	0.77	128	**0.52**	**86**	**0.34**	**57**	**0.26**	**43**
Sulphur amino acids	0.60♦	2.76 methionine and 0.43 cystine	0.65	108	**0.44**	**73**	**0.29**	**48**	**0.22**	**36**
Isoleucine	0.50	4.56	0.93	186	0.62	125	**0.41**	**83**	**0.31**	**62**
Leucine	0.75	8.75	1.78	238	1.20	160	0.80	106	**0.59**	**79**
phenylalanine and tyrosine•	0.80	4.48 PHN + 4.78 TYR	1.83	228	1.23	153	0.82	102	**0.61**	**76**
lysine	0.70	7.98	1.63	232	1.09	156	0.72	104	**0.54**	**78**
Histidine	0.30	2.39	0.49	162	0.33	109	**0.22**	**72**	**0.16**	**54**
Arginine	0.60	3.48	0.71	118	**0.48**	**79**	**0.32**	**53**	**0.24**	**39**
tryptophan	0.15	1.07	0.22	146	**0.15**	**98**	**0.10**	**65**	**0.07**	**48**
non-essential amino acids (mixture of Glycine (G), Alanine (A) and Serine (S)	0.59	G =1.72 A=2.88 S=5.19	1.99	338	1.34	227	0.89	151	0.66	113

* National research council. National Academy of Sciences, nutrition requirement of laboratory rats. Third edition 1978.

♣ Special diets services (1995).

♦ One-third to one-half can be supplied by L-cystine.

• One-third to one-half of phenylalanine requirement can be supplied by L-tyrosine. The percentage of phenylalanine requirement for growth present in the 180 g, 120 g, 80 g, and 60 g/kg diet using casein as a source of protein based on the assumption that half of this requirement supply by tyrosine.

E: No significant difference in the energy content (18 ± 0.34 kJ/g dried food) was found between all the diets used in the thesis. The energy content of diet was measured using ballistic bomb calorimetry (Miller & Payne, 1959).

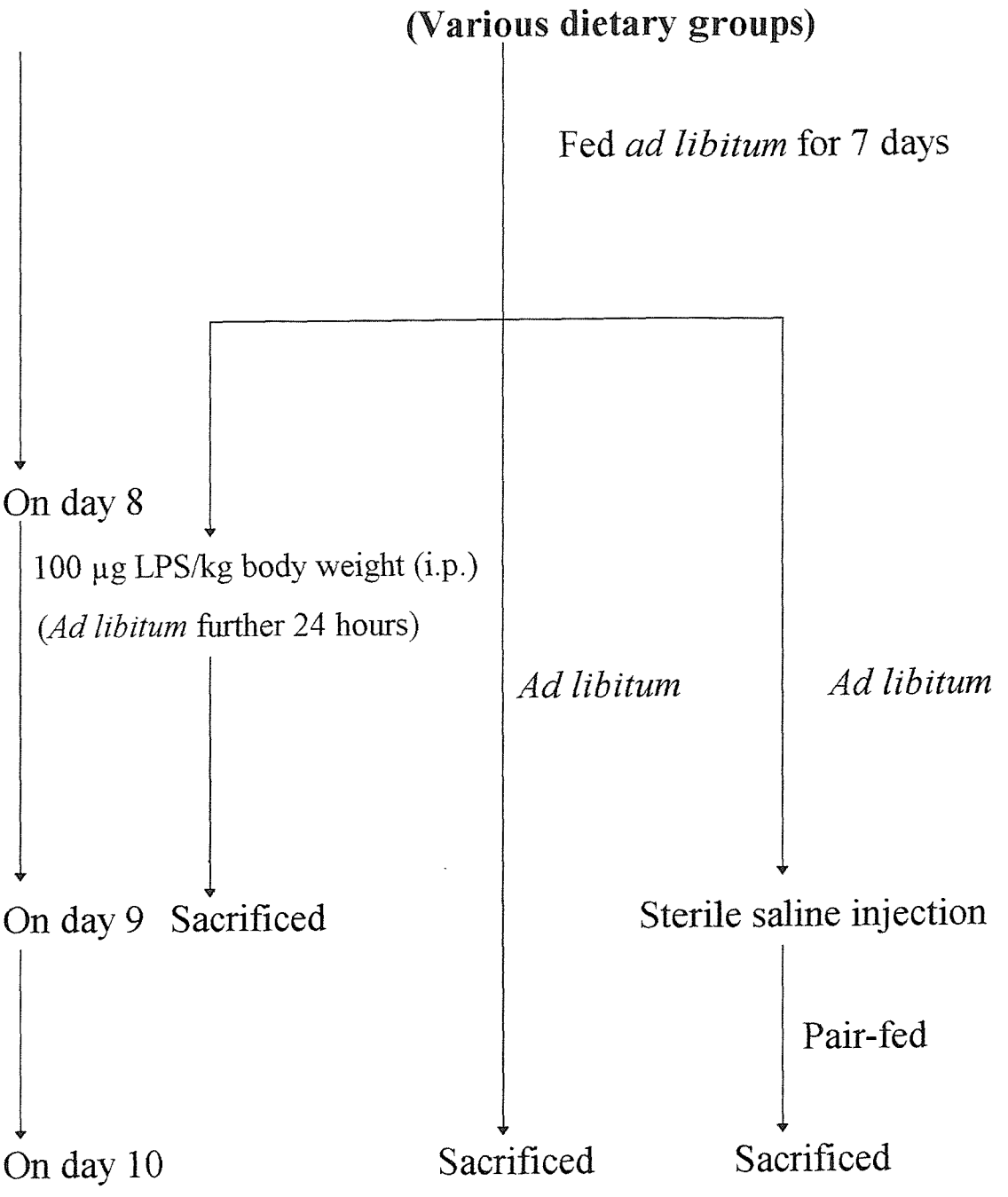
4.3 Experimental protocol

Male weanling wistar rats from Southampton University Medical School Colony weighing 50-70 g were housed individually in plastic metabolic cages and were maintained at 22 ± 1°C with a 12-12 hour light-dark cycle (Lights on at 7:30 AM).

Rats were divided into four different dietary groups; 18 rats/group and fed *ad libitum* the semi-synthetic diets for 7 days. On day 8, animals from each group were subdivided into three treatment groups. One third of each group was injected with 100 μg/kg body weight lipopolysaccharide (LPS) from Escherichia coli (Sigma E.coli, trichloroacetic acid extraction, strain 0127:B8) in sterile non-pyrogenic saline, intraperitoneally (i.p.). These animals were allowed free access to food for a further 24 hours. On day 9, one-third of each group received an i.p. injection of a sterile non-pyrogenic saline (1ml saline/kg body weight), and was pair-fed with an amount of diet similar to that consumed by LPS-treated animals for the 24 hours after injection. The final one-third of each group continued to feed *ad libitum*. LPS-treated animals were killed on day 9, and the rest of the animals were killed on day 10. See Figure 4-1 for the schematic representation of the experimental protocol. Previous studies carried out in our laboratory have shown that feeding low-protein diet for eight days was enough to change the amino acid pool, and reduced the weight gain of the rats, but it will not lead to the animals suffering from serious protein malnutrition or other illness (Hunter & Grimble, 1994; Hunter & Grimble, 1997). Das & Waterlow (1974) have shown that the adaptation of urea-nitrogen excretion, urea-cycle enzymes, and the enzymes necessary to make the amino group available for entry into the urea cycle (e.g. glutamate dehydrogenase, aspartate aminotransferase and alanine aminotransferase) to different levels of protein intake occurred in 30 hours after the diet has been changed from low-protein diet to high protein diet, or vice versa. Furthermore, Hum *et al.* (1992) have shown that feeding low casein diets for seven days was enough to cause changes in body and liver weights, in hepatic glutathione concentration, and in plasma urea, methionine, cysteine and glutathione concentrations.

All rats were killed by stunning and decapitation. The liver, lung, spleen, and thymus from each rat were rapidly dissected out, cleaned, and weighed. Prior to snap freezing in liquid nitrogen, small samples were taken from liver, lung, spleen and thymus for immediate analyses of glutathione content. Small samples of lung tissues were also dissected from rats, and then immediately preserved in neutral buffered formalin for histology, to count neutrophilic cells in lung. All the above organs were then stored at -80°C for further analysis. Blood was collected in clean centrifuge tube, allowed to clot, then centrifuged at 2000 rpm/min for 15 minutes at 4°C. The serum was collected, and stored at -20°C.

Figure 4-1 Schematic representation of the experimental protocol



4.4 Results

4.4.1 Growth, food intake, sulphur amino acid intake, and organ weights

Average daily weight gain, food and sulphur amino acid intake, and food, protein and energy efficiency before starting treatments

Table 4.3 shows that there was a progressive reduction in the average daily weight gain as dietary protein was decreased. Although the average daily food intake was similar between the groups, there was a progressive reduction in the food efficiency (g weight gain/g food intake) and the energy efficiency (mg weight gain/kJ eaten) as dietary protein content was decreased. The result of the average daily sulphur amino acid intake shows that the NP group consumed the highest amount of sulphur amino acids, followed by the MP, LP and VLP groups, respectively (Table 4.3). The amount of sulphur amino acid intake recommended to support growth in growing rats is between 5-6 g/kg of diet (National Research Council, 1978). The results indicate that sulphur amino acids may be important in supporting growth process, as they were present in adequate amount in the NP group {6.5 g (45 mmol)/kg diet}, in a moderate amount in the MP group {4.3 g (30 mmol)/kg diet}, in a low amount in the LP group {2.9 g (20 mmol)/kg diet}, and in a very low amount in the VLP group {2.1 g (15 mmol)/kg diet}. When the protein concentration in the diet was decreased from 180 g to 80 g/kg diet, protein efficiency, the efficiency with which dietary protein was used for growth (g weight gain/g protein eaten), was increased. This was statistically significant when comparing animals fed the MP and the LP diets with animals fed the NP diet. Contrarily, further reduction in protein concentration (60 g/kg diet) did not increase the protein efficiency and the protein efficiency was significantly lower than in the LP group.

Table 4.3 Average daily weight gain, food and sulphur amino acid intake, and food, protein and energy efficiency of rats fed diets of graded casein content, before starting treatments

Dietary groups	NP	MP	LP	VLP	one-way ANOVA	
g protein/kg diet	180	120	80	60	p-value	F ratio
weight gain (g)	6.1 ± 0.26^{a}	5.1 ± 0.37^{b}	3.6 ± 0.22^{c}	2.1 ± 0.18^{d}	p=0.000	F=42.4
Food intake (g)	12.3 ± 0.28^{a}	12.2 ± 0.43^{a}	11.9 ± 0.37^{a}	11.1 ± 0.43^{a}	p=0.117	F=2.03
Sulphur amino acid intake (µmol)	554 ± 12^{a}	368 ± 13^{b}	238 ± 7^{c}	167 ± 6^{d}	p=0.000	F=279.4
Weight gain/ food intake (g/g)	0.49 ± 0.02^{a}	0.42 ± 0.03^{b}	0.31 ± 0.01^{c}	0.19 ± 0.01^{d}	p=0.000	F=46.5
Weight gain /energy intake (mg/kJ)	27.3 ± 0.98^{a}	24.4 ± 1.60^{b}	17.0 ± 0.83^{c}	10.5 ± 0.71^{d}	p=0.000	F=46.5
weight gain/protein intake (g/g)	2.7 ± 0.10^{a}	3.5 ± 0.24^{bc}	3.8 ± 0.19^{c}	3.1 ± 0.21^{ab}	p=0.000	F=6.1

* Results are presented as mean ± standard error of the mean, n=18 per group. Means within each row having different letter superscripts following the number differ significantly.

The Effect of LPS on food and sulphur amino acid intake

The effect of LPS on food and sulphur amino acid intake can be found in Table 4.4. In animals fed *ad libitum*, the food intake was similar between the groups. In the LPS-treated, and pair-fed controls, a reduction in food intake occurred in all animals in the various groups, and this reduction was statistically significant when compared to values from animals fed *ad libitum*.

In animals fed *ad libitum*, there was a progressive reduction in sulphur amino acid intake as dietary protein content was decreased. The diets caused a three-fold variation in intake of these amino acids. In the LPS-treated, and pair-fed controls,

sulphur amino acid intake fell further, due to the reduction in food intake, and there was a trend for a reduction in sulphur amino acid intake as dietary protein content was decreased between the NP group and the VLP group. The difference was statistically significant when comparing the NP group with the LP and the VLP groups.

Table 4.4 Effect of LPS on food intake and sulphur amino acid intake of rats fed diets of graded casein content

Dietary groups	NP	MP	LP	VLP	two-way ANOVA	
g protein/kg diet	180	120	80	60	p-value	F ratio
Food intake (g/day)						
ad libitum	13.0 ± 0.78 [a]	12.0 ± 0.60 [a]	11.9 ± 0.69 [a]	12.3 ± 0.42 [a]	D=0.493	F=0.81
pair-fed	7.9 ± 0.86 [a]*	6.6 ± 1.22 [a]*	7.6 ± 0.68 [a]*	8.3 ± 0.82 [a]*	T=0.000	F=23.04
LPS	8.2 ± 1.38 [a]*	7.3 ± 1.81 [a]*	7.7 ± 1.37 [a]*	8.9 ± 1.24 [a]*	I=0.990	F=0.14
Sulphur amino acid intake (μmol/day)						
ad libitum	586 ± 35 [a]	360 ± 18 [b]	238 ± 14 [c]	184 ± 6 [d]	D=0.000	F=46.69
pair-fed	355 ± 39 [a]*	198 ± 36 [b]*	152 ± 14 [b]*	124 ± 12 [b]*	T=0.000	F=20.76
LPS	368 ± 62 [a]*	218 ± 54 [ab]*	154 ± 27 [b]*	134 ± 19 [b]*	I=0.119	F=1.78

-Results are presented as mean ± standard error of the mean, n=6 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

Final body weight

The result for final body weight can be seen in Table 4.5. In animals fed *ad libitum*, the final body weight in the VLP group was significantly lower than in the NP and the MP group. In the pair-fed controls, the final body weight was significantly lower in animals fed the LP and the VLP diets than in animals fed the NP diet. In animals fed the VLP diet, the final body weight was also lower than in animals fed the MP diet. In the LPS-treated animals, the LP and the VLP dietary groups had lower final body weight compared with other dietary groups. The difference was statistically significant when comparing the LP group with the NP and the MP groups.

Comparing the LPS-treated rats, animals fed *ad libitum* and pair-fed controls, it can be seen that in the NP group, the mean value was lower in the LPS-treated animals than in the pair-fed and *ad libitum* fed animals, but this difference was not statistically significant. In the MP and the LP groups, the final body weights were significantly lower in the LPS-treated animals compared with the animals fed *ad libitum*. In the VLP group, body weights were significantly lower in the pair-fed animals compared with the animals fed *ad libitum*.

Table 4.5 Final body weight of rats fed diets of graded casein content

Dietary groups	NP	MP	LP	VLP	two-way ANOVA	
g protein/kg diet	180	120	80	60	p-value	F ratio
Final body weight (g)						
ad libitum	94.9 ± 2.7 ^{a}	97.9 ± 4.4 ^{a}	87.2 ± 3.0 ^{ab}	79.8 ± 2.4 ^{b}	D=0.000	F=15.66
pair-fed	98.3 ± 5.4 ^{a}	86.2 ± 5.3 ^{ab}	79.6 ± 3.3 ^{bc}	69.4 ± 3.5 ^{c}*	T=0.000	F=10.65
LPS	83.8 ± 4.1 ^{a}	83.1 ± 2.8 ^{a}*	72.7 ± 2.0 ^{b}*	71.5 ± 4.2 $^{a\,b}$	I=0.300	F=1.24

-Results are presented as mean ± standard error of the mean, n=6 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

Organ weights

a) Total liver weight

Table 4.6 shows that in animals fed *ad libitum*, the total liver weight was significantly lower in the VLP group compared with the NP and the MP groups. In the pair-fed controls, the total liver weight in the VLP group was significantly lower than in the other dietary groups. In the LPS-treated animals, the total liver weight in animals fed the LP and the VLP diets was significantly lower than in animals fed the NP and the MP protein diets, and it was lower in the VLP group compared with the LP group.

Due to treatments, the total liver weight in the pair-fed controls given the MP, LP, and the VLP diets was significantly lower than in the *ad libitum* fed animals. LPS-treated animals fed the MP and the VLP diets had higher total liver weight compared with the corresponding pair-fed controls.

b) Relative liver weight

In animals fed *ad libitum*, no difference in the relative liver weight (g/kg body weight) was found between the groups. In the pair-fed animals, it was significantly lower in the VLP group compared with the LP group. In the LPS-treated animals, the relative liver weight was significantly lower in the VLP group compared with other dietary groups.

Due to treatments, in all dietary groups, the relative liver weight in the LPS-treated animals was significantly higher than in the pair-fed controls. The relative liver weight in LPS-treated animals fed the MP and the LP diets was also higher than in animals fed *ad libitum*. In the NP, and the VLP group, the relative liver weight in the pair-fed animals was significantly lower than in the animals fed *ad libitum* (Table 4.6).

c) Total lung weight

In animals fed *ad libitum*, total lung weight was significantly lower in the VLP group compared with the NP and the MP groups. In the pair-fed animals, it was

higher in the NP group compared with other dietary groups. This difference was not statistically significant due to large inter-group variability. In the LPS-treated animals, it was lower in the LP and the VLP groups compared with the NP and the MP groups. This was statistically significant when comparing the LP group with the NP and the MP groups.

No significant difference in total lung weight was found between the groups due to treatments (Table 4.6).

d) Relative lung weight

Due to diet, no significant difference in the relative lung weight (g/kg body weight) was found between the groups. Due to treatments, in the MP and the LP groups, relative lung weight was significantly higher in the LPS-treated animals compared with the corresponding pair-fed and *ad libitum* fed animals (Table 4.6). In the NP group, there was a tendency for an increase in the relative lung weight after LPS treatment compared with the pair-fed controls, but the increase was not statistically significant.

e) Total spleen weight

In animals fed *ad libitum*, there was a significant reduction in the total spleen weight as dietary protein content was decreased between the NP and the LP groups. The extent of the reduction in the total spleen weight was greater between the MP and the LP groups. Further reduction in the total spleen weight occurred in the VLP group. This reduction was not statistically significant, when compared with the LP group. In the pair-fed animals, the total spleen weight in the LP and the VLP groups was significantly lower than in the NP group. In the LPS-treated animals, total spleen weight in the LP and VLP groups was significantly lower than in the NP and MP groups.

No difference in the total spleen weight was found between the groups due to treatments (Table 4.6).

f) Relative spleen weight

In the animals fed *ad libitum*, the relative spleen weight (g/kg body weight) was significantly lower in the LP and VLP groups compared with other dietary groups. In the pair-fed animals, the mean values in the NP and MP group was higher than in the LP and VLP groups, but this difference was not statistically significant. In the LPS-treated animals, the relative spleen weight was significantly lower in the VLP group compared with the NP and the MP groups.

Due to treatments, the relative spleen weight in the VLP group was higher in the LPS treated animals compared with the corresponding animals fed *ad libitum* (Table 4.6).

g) Total thymus weight

In contrast to spleen, no significant difference in the total thymus weight was found between the groups in the animals fed *ad libitum* and in LPS-treated animals. In the pair-fed animals, total thymus weight was lower in the VLP group compared with the NP group.

Due to treatments, the pair-fed and LPS-treated animals fed the MP diet had lower total thymus weight compared with *ad libitum* fed animals (Table 4.6).

h) Relative thymus weight

Neither diet nor treatments caused a significant difference in the relative thymus weight (g/kg body weight) between the groups (Table 4.6).

Table 4.6 Organ weights of rats fed diets of graded casein content, after starting treatments

Dietary groups	NP	MP	LP	VLP	two-way ANOVA	
g protein/kg diet	180	120	80	60	p-value	F ratio
Liver weight (g)						
ad libitum	4.54 ± 0.21 ^{a}	4.50 ± 0.27 ^{a}	3.98 ± 0.17 ^{ab}	3.6 ± 0.13^{b}	D=0.000	F=20.71
pair-fed	4.01 ± 0.28 ^{a}	3.55 ± 0.32 ^{a}*	3.35 ± 0.17 ^{a}*	2.69 ± 0.15^{b}*	T=0.000	F=17.10
LPS	4.40 ± 0.11 ^{a}	4.60 ± 0.12 ^{a}♠	3.8 ± 0.14^{b}	3.30 ± 0.13^{c}♠	I=0.614	F=0.75
Relative liver weight (g/kg body weight)						
ad libitum	47.9 ± 2.2 ^{a}	46.0 ± 2.4 ^{a}	45.7 ± 1.6 ^{a}	45.4 ± 1.0 ^{a}	D=0.019	F=3.59
pair-fed	40.7 ± 1.7 ^{ab}*	40.9 ± 2.1 ^{ab}	42.1 ± 1.2 ^{a}	38.7 ± 0.8^{b}*	T=0.000	F=43.0
LPS	52.6 ± 1.5 ^{a}♠	55.5 ± 2.3 ^{a}♠*	52.0 ± 1.0 ^{a}♠*	46.2 ± 1.3 ^{b}♠	I=0.215	F=1.44
Lung weight (mg)						
ad libitum	753 ± 34 ^{ab}	774 ± 25 ^{a}	670 ± 27^{bc}	629 ± 32^{c}	D=0.000	F=8.21
pair-fed	804 ± 68 ^{a}	664 ± 68 ^{a}	638 ± 16 ^{a}	620 ± 55 ^{a}	T=0.637	F=0.45
LPS	746 ± 35 ^{a}	731 ± 13 ^{a}	634 ± 18 ^{c}	636 ± 40^{ac}	I=0.574	F=0.80
Relative lung weight (g/kg body weight)						
ad libitum	7.92 ± 0.19 ^{a}	7.95 ± 0.30 ^{a}	7.69 ± 0.18 ^{a}	7.90 ± 0.36 ^{a}	D=0.494	F=0.81
pair-fed	8.20 ± 0.53 ^{a}	7.66 ± 0.25 ^{a}	8.04 ± 0.16 ^{a}	8.91 ± 0.61 ^{a}	T=0.001	F=8.54
LPS	8.96 ± 0.47 ^{a}	8.82 ± 0.19 ^{a}♠*	8.94 ± 0.29 ^{a} ♠*	8.93 ± 0.43 ^{a}	I=0.676	F=0.67
Spleen weight (mg)						
ad libitum	528 ± 44 ^{a}	454 ± 19 ^{a}	290 ± 19^{b}	261 ± 19^{b}	D=0.000	F=30.25
pair-fed	488 ± 51 ^{a}	371 ± 58 ^{ab}	307 ± 29^{b}	261 ± 21^{b}	T=0.352	F=1.06
LPS	468 ± 20 ^{a}	459 ± 26 ^{a}	331 ± 34^{b}	294 ± 16^{b}	I=0.467	F=0.95
Relative spleen weight (g/kg body weight)						
ad libitum	5.55 ± 0.43 ^{a}	4.64 ± 0.10 ^{a}	3.31 ± 0.14^{b}	3.28 ± 0.23 ^{b}	D=0.000	F=13.5
pair-fed	4.98 ± 0.51 ^{a}	4.25 ± 0.53 ^{a}	3.84 ± 0.29 ^{a}	3.77 ± 0.31 ^{a}	T=0.003	F=6.61
LPS	5.62 ± 0.25 ^{a}	5.55 ± 0.37 ^{a}	4.69 ± 0.54 $^{a\,b}$	4.17 ± 0.29 ^{b}*	I=0.437	F=1.00
Thymus weight (mg)						
ad libitum	546 ± 40 ^{a}	626 ± 40 ^{a}	526 ± 28 ^{a}	500 ± 40 ^{a}	D=0.014	F=3.8
pair-fed	582 ± 39 ^{a}	471 ± 41 ^{ab}*	504 ± 37 ^{ab}	392 ± 39^{b}	T=0.004	F=6.1
LPS	477 ± 56 ^{a}	472 ± 33 ^{a}*	461 ± 29 ^{a}	421 ± 32 ^{a}	I=0.225	F=1.4
Relative thymus weight (g/kg body weight)						
ad libitum	5.76 ± 0.40 ^{a}	6.64 ± 0.47 ^{a}	6.03 ± 0.24 ^{a}	6.26 ± 0.45 ^{a}	D=0.467	F=0.8
pair-fed	5.96 ± 0.34 ^{a}	5.05 ± 0.36 ^{a}	6.36 ± 0.49 ^{a}	5.62 ± 0.38 ^{a}	T=0.305	F=1.2
LPS	5.69 ± 0.60 ^{a}	5.66 ± 0.25 ^{a}	6.31 ± 0.31 ^{a}	5.88 ± 0.27 ^{a}	I=0.265	F=1.3

-Results are presented as mean ± standard error of the mean, n=6 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

4.4.2 Glutathione concentration, and glutathione peroxidase and reductase activities

Glutathione concentration

a) Hepatic glutathione concentration

Dietary protein intake, food intake and LPS treatment influenced hepatic glutathione (GSH) concentration (Table 4.7).

In animals fed *ad libitum*, GSH concentration in liver was significantly higher in rats fed the NP diet compared with other groups fed the MP, LP and VLP diets, and it was higher in the MP group compared with the LP and the VLP groups. No further reduction in GSH concentration was found when the protein concentration in the diet was decreased from 80 g to 60 g/kg diet. Although the average daily weight gain of rats fed the LP diet was significantly higher than in rats fed the VLP diet, no difference in GSH concentration was found between the LP and VLP groups. In the LPS-treated animals, GSH concentration of the NP group was significantly higher and to a greater extent than in the rest of the groups. LPS-treated animals fed the LP diet were able to increase GSH concentration in liver to be significantly higher than in the VLP group. GSH concentration of LPS-treated animals fed the MP diet was significantly higher than in LPS-treated animals fed the LP and the VLP diets. Restricting food intake by pair feeding resulted in a decrease in GSH concentration in the NP and MP groups, the LP and VLP group were unaffected. In the pair-fed animals, GSH concentration was higher in the NP group compared with other dietary groups fed the MP, LP, and the VLP diets. No significant difference was found in hepatic GSH concentration between the MP, LP and the VLP groups.

Comparing the LPS-treated animals with pair-fed controls, it can see that the NP, MP and LP animals were able to increase hepatic GSH concentration after LPS treatment compared with the pair-fed controls, while LPS-treated animals fed the VLP diet were unable to increase GSH concentration.

b) Lung glutathione concentration

Lung GSH concentration was influenced by dietary protein content, food intake and LPS treatment, but to a less extent than in liver (Table 4.7).

In animals fed *ad libitum*, GSH concentration in the NP group was significantly higher than in the MP, LP and the VLP groups. No difference was found in lung GSH concentration between the groups fed the MP, LP and VLP diets. Restricting food intake by pair feeding resulted in a fall in GSH only in the NP group. In the pair-fed animals, no difference was found in GSH concentration between animals fed the low-protein diets (the LP and VLP diets) and animals fed the NP and the MP diets. Animals fed the MP diet had lower GSH concentration than animals fed the NP diet. After LPS treatment, lung GSH concentration in the NP, MP and LP groups was significantly higher than in the VLP group, and it was higher in the NP and the MP groups compared with the LP group. GSH concentration in lung was similar between the NP and the MP groups.

Comparing the LPS-treated animals with pair-fed controls, it can be seen that animals fed the NP and the MP diets were able to increase lung GSH concentration after LPS treatment compared with the corresponding pair-fed controls. However, in the LP group, GSH concentration did not increase after LPS treatment, and in the VLP group, GSH concentration of the LPS-treated animals was lower than in the pair-fed controls. Thus the nature of the response of lung GSH to inflammation is greatly affected by diet protein content.

c) Spleen glutathione concentration

Spleen GSH concentration was influenced by dietary protein content, and LPS treatment, but to a lesser extent than in liver (Table 4.7).

In animals fed *ad libitum*, GSH concentration in spleen was similar between the groups fed the NP, MP and the LP diets, and it was significantly lower in the VLP group compared with the NP and MP groups. Unlike in liver and lung, restricting food intake by pair feeding had no effect on GSH concentration. In the LPS-treated animals, GSH concentration in spleen was significantly higher in the NP group

compared with the LP and VLP groups. GSH concentration of the VLP group was significantly lower than in the NP, MP, and the LP groups.

Comparing the LPS-treated animals with pair-fed controls, it can be seen that in the VLP group, spleen GSH concentration was lower in the LPS-treated animals than in the pair-fed controls. This reduction was due to the combined interaction between the diet, which is severely deficient in protein, and treatments. No statistically significant difference was found between the LPS-treated animals and pair-fed controls in the NP, MP and the LP groups.

d) Thymus glutathione concentration

Animals fed the NP, MP and the LP diets were able to maintain thymus GSH concentration under abnormal conditions, such as after receiving LPS or under food restriction (Table 4.7).

In animals fed *ad libitum*, GSH concentration in thymus was significantly lower in the LP and the VLP groups compared with the NP and the MP groups. In the pair-fed controls, no difference in thymus GSH concentration was found between the groups. After LPS treatment, thymus GSH concentration was significantly lower in the VLP group compared with other groups.

As in lung and spleen, animals fed the VLP diet failed to enhance GSH concentration in thymus after LPS treatment, and it was significantly lower than the corresponding pair-fed controls.

Table 4.7 Glutathione concentration in liver, lung, spleen and thymus of rats fed diets of graded casein content, after starting treatments (μmol/g tissue)

Dietary groups	NP	MP	LP	VLP	two-way ANOVA	
g protein/kg diet	180	120	80	60	p-value	F ratio
Liver						
ad libitum	5.50 ± 0.36^{a}	2.28 ± 0.12^{b}	1.58 ± 0.11^{c}	1.53 ± 0.16^{c}	D= 0.000	F=141
pair-fed	2.99 ± 0.37^{a}*	1.42 ± 0.18^{b}*	1.79 ± 0.10^{b}	1.78 ± 0.08^{b}	T= 0.000	F=40.4
LPS	6.33 ± 0.41^{a}♠	2.92 ± 0.07^{b}♠*	2.42 ± 0.16^{c}♠*	1.86 ± 0.12^{d}	I= 0.000	F= 12.9
Lung						
ad libitum	1.79 ± 0.07^{a}	1.48 ± 0.11^{b}	1.24 ± 0.04^{b}	1.27 ± 0.08^{b}	D= 0.000	F= 14.7
pair-fed	1.50 ± 0.06^{a}*	1.24 ± 0.07^{b}	1.50 ± 0.11^{ab}	1.47 ± 0.13^{ab}	T= 0.392	F=0.95
LPS	1.80 ± 0.06^{a}♠	1.73 ± 0.04^{a}♠	1.40 ± 0.09^{b}	1.08 ± 0.02^{c}♠	I= 0.000	F=6.97
Spleen						
ad libitum	2.91 ± 0.12^{a}	2.59 ± 0.13^{a}	2.44 ± 0.25^{ab}	2.11 ± 0.16^{b}	D=0.000	F=17
pair-fed	2.99 ± 0.15^{a}	2.28 ± 0.08^{b}	2.70 ± 0.15^{ac}	2.42 ± 0.09^{bc}	T=0.643	F=0.44
LPS	3.05 ± 0.11^{a}	2.80 ± 0.16^{ab}	2.45 ± 0.11^{b}	2.03 ± 0.06^{c}♠	I=0.048	F=2.28
Thymus						
ad libitum	1.93 ± 0.06^{a}	1.92 ± 0.05^{a}	1.62 ± 0.06^{b}	1.68 ± 0.09^{b}	D=0.001	F=8.54
pair-fed	2.04 ± 0.13^{a}	1.79 ± 0.05^{a}	1.90 ± 0.18^{a}	1.91 ± 0.04^{a}	T=0.047	F=3.20
LPS	2.00 ± 0.10^{a}	1.93 ± 0.06^{a}	1.77 ± 0.06^{a}	1.37 ± 0.06^{b}♠*	I=0.003	F=3.58

-Results are presented as mean ± standard error of the mean, n=6 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

Glutathione peroxidase activity in liver, lung, spleen, and thymus

a) Glutathione peroxidase activity in liver

The results for the liver glutathione perxidase (GPx) can be seen in (Table 4.8). Feeding a diet severely deficient in protein caused a significant increase in GPx activity in liver. The activity of GPx was altered by LPS treatment and restriction in food intake, if the animals fed a diet containing 120 g protein or less/kg of diet. The fall in food intake, which occurred by pair feeding, led to a fall in GPx activity.

However, at the same level of intake, the introduction of the inflammatory challenge of LPS restored GPx values to those seen in the groups fed *ad libitum*.

In the *ad libitum* fed and in LPS-treated animals, GPx activity in animals fed the VLP diet was significantly higher than in other dietary groups. In the pair-fed animals, GPx activity was similar between the groups. Comparing the pair-fed controls, LPS-treated rats and *ad libitum*-fed animals, it can be seen that GPx activity of the pair-fed animals in the MP, LP, and VLP groups was lower than the corresponding *ad libitum* fed controls. GPx activity in the LPS-treated animals fed the MP, LP and VLP diets was significantly higher than in the pair-fed animals. In the NP group, no difference in GPx activity was found due to treatments.

b) Glutathione peroxidase activity in lung

The results for lung GPx activity can be found in (Table 4.8). There was no difference in GPx activity between the *ad libitum* fed groups. In the pair-fed animals, GPx activity was higher in the VLP group compared with other groups. In the LPS- treated animals, GPx activity in the VLP group was higher than in the NP and MP groups.

No difference in GPx activity between the groups was found due to treatments.

c) Glutathione peroxidase activity in spleen and thymus

Neither diet nor treatments caused a significant alteration in the activity of GPx in spleen and thymus (Table 4.8).

Table 4.8 Glutathione peroxidase activity in liver, lung, spleen and thymus of rats fed diets of graded casein content, after starting treatments (units/gram protein)

Dietary groups	NP	MP	LP	VLP	two-way ANOVA
g protein/kg diet	180	120	80	60	p-value F ratio
Liver					
ad libitum	38.5 ± 2.62[a]	40.1 ± 2.82[a]	42.0 ± 2.97[a]	48.1 ± 1.64[b]	D= 0.046 F=2.9
pair-fed	35.0 ± 2.95[a]	30.0 ± 2.19[a]*	27.0 ± 1.90[a]*	30.5 ± 1.54[a]*	T=0.000 F=28
LPS	38.6 ± 2.31[a]	39.8 ± 3.03[a]♠	40.6 ± 2.17[a]♠	45.9 ± 1.63[b]♠	I=0.100 F=1.9
Lung					
ad libitum	57.4 ± 2.11[a]	55.5 ± 1.90[a]	60.9 ± 0.93 [a]	60.9 ± 1.23[a]	D=0.000 F=13
pair-fed	53.6 ± 2.54[a]	54.1 ± 3.8[a]	57.7 ± 2.20[a]	66.7 ± 1.10[b]	T=0.28 F=1.32
LPS	55.2 ± 2.8[a]	56.8 ± 2.65[a]	62.0 ± 1.34[ab]	67.7 ± 2.3[b]	I=0.31 F=1.23
Spleen					
ad libitum	57.5 ± 2.35 [a]	57.6 ± 2.01 [a]	59.7 ± 1.27 [a]	60.5 ± 3.76 [a]	D=0.545 F=0.7
pair-fed	55.0 ± 0.62 [a]	56.8 ± 2.12 [a]	57.4 ± 1.75 [a]	61.9 ± 1.04 [a]	T=0.673 F=0.4
LPS	58.2 ± 2.24 [a]	58.9 ± 1.48 [a]	58.8 ± 1.73 [a]	54.9 ± 1.67 [a]	I=0.214 F=1.5
Thymus					
ad libitum	52.7 ± 3.51 [a]	53.06 ± 1.81 [a]	58.8 ± 4.25 [a]	59.6 ± 2.68 [a]	D=0.088 F=2.3
pair-fed	52.9 ± 5.25 [a]	54.11 ± 4.15 [a]	55.5 ± 4.14 [a]	59.7 ± 2.65 [a]	T=0.382 F=1.0
LPS	58.9 ± 1.68 [a]	55.20 ± 0.92 [a]	58.3 ± 1.85 [a]	61.3 ± 1.73 [a]	I=0.940 F=0.28

-Results are presented as mean ± standard error of the mean, n=4 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

Glutathione reductase activity in liver, lung, spleen, and thymus

a) Glutathione reductase activity in liver

Glutathione reductase (GR) activity in liver was influenced by dietary protein intake in animals fed *ad libitium* and in LPS-treated animals (Table 4.9).

In animals fed *ad libitum* and in LPS-treated animals, GR activity in liver was significantly higher in animals fed the VLP diet compared with animals fed the NP, MP, and LP diets. Restricting food intake by pair feeding prevented this effect so that no significant difference in GR activity was found between any of the groups.

Unlike food restriction, LPS treatment retained the activity of GR in animals fed the VLP diet to values seen in the *ad libitum* animals.

b) Glutathione reductase activity in lung, spleen, and thymus

In all of the above organs, no significant difference in GR activity was found between the groups due to the diet or treatments (Table 4.9).

Table 4.9 Glutathione reductase activity in liver, lung, spleen and thymus of rats fed diets of graded casein content, after starting treatments (units/mg protein)

Dietary groups	NP	MP	LP	VLP	two-way ANOVA
g protein/kg diet	180	120	80	60	p-value F ratio
Liver					
ad libitum	17.1 ± 3.24 [a]	17.0 ± 3.41 [a]	16.8 ± 2.62 [a]	30.2 ± 2.53 [b]	D=0.000 F=10.12
pair-fed	14.5 ± 3.02 [a]	16.6 ± 2.42 [a]	17.1 ± 1.27 [a]	19.0 ± 3.70 [a]	T=0.105 F=2.40
LPS	15.2 ± 1.90[a]	17.7 ± 1.67 [a]	19.0 ± 1.60[a]	29.6 ± 3.43[b]	I=0.283 F=1.30
Lung					
ad libitum	18.7 ± 1.36 [a]	18.5 ± 3.02 [a]	20.2 ± 2.71 [a]	18.8 ± 0.46 [a]	D=0.497 F=0.81
pair-fed	16.8 ± 1.54 [a]	16.8 ± 1.96 [a]	17.4 ± 2.19 [a]	20.8 ± 2.66 [a]	T=0.444 F=0.83
LPS	17.4 ± 1.94 [a]	16.3 ± 0.95 [a]	16.3 ± 2.32 [a]	19.2 ± 0.46 [a]	I=0.899 F=0.36
Spleen					
ad libitum	17.1 ± 2.71 [a]	16.5 ± 0.64 [a]	18.7 ± 1.71 [a]	18.8 ± 0.90 [a]	D=0.58 F=0.66
pair-fed	17.3 ± 0.62 [a]	21.7 ± 0.33 [a]	16.7 ± 0.40 [a]	16.9 ± 0.85 [a]	T=0.180 F=1.79
LPS	14.9± 2.39 [a]	15.5 ± 2.28 [a]	17.4 ± 0.92 [a]	17.6 ± 0.68 [a]	I=0.126 F=1.80
Thymus					
ad libitum	14.9 ± 1.44 [a]	15.7 ± 2.08 [a]	15.7 ± 1.08 [a]	19.5 ± 0.98 [a]	D=0.180 F=1.73
pair-fed	14.1 ± 0.85 [a]	15.5 ± 1.19 [a]	16.5 ± 1.33 [a]	13.9 ± 2.29 [a]	T=0.411 F=0.91
LPS	14.5 ± 1.67 [a]	14.5 ± 2.00 [a]	14.1 ± 0.77 [a]	18.3 ± 1.86 [a]	I=0.311 F=1.24

-Results are presented as mean ± standard error of the mean, n=4 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly (P< 0.05%). * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair - fed and LPS - treated groups.

4.4.3 Protein in liver and lung after starting treatments

Tissue protein is reported in three ways, as concentration, as total amount per organ and as total amount related to body weight.

Liver protein concentration

Hepatic protein concentration was severely affected when the protein concentration in the diet was as low as 60 g/kg (the VLP group) (Table 4.10).

In animals fed *ad libitum*, hepatic protein concentration in the VLP group was significantly lower than in other groups. In the pair-fed animals, no significant difference in the protein concentration was found between the groups. After LPS treatment, animals fed the LP and the VLP diets had a lower protein concentration than the NP and the MP groups. This reduction become larger as the protein concentration in the diet was decreased.

Comparing the LPS-treated rats, animals fed *ad libitum* and pair-fed controls, it can be seen that in the VLP group, hepatic protein concentration was higher in the pair-fed controls, when compared with the *ad libitum* and LPS-treated animals. In the LP group, it was higher in the pair-fed controls compared with the LPS-treated animals. The increase in protein concentration occurred in the pair-fed animals may be due to a reduction in other chemical constituents of the liver and may be caused by the breakdown of glycogen, loss of water, and/or increase lipolysis.

Total liver protein

The total liver protein content was mainly influenced by dietary protein intake. In general, the total liver protein among animals of various dietary and treatment groups varied due to the difference in the size of livers (see Table 4.6 & Table 4.10).

In animals fed *ad libitum*, total liver protein was significantly lower in the VLP group compared with other groups, and it was significantly lower in the LP group compared with the NP group. In the pair-fed animals, the total liver protein was significantly lower in the VLP group compared with the NP and the MP groups. In

the LPS-treated animals, total hepatic protein in the LP and VLP animals was significantly lower than in the NP and MP animals.

Comparing the LPS treated animals, pair-fed and *ad libitum* fed animals, it can be seen that in the MP group, the pair-fed animals had a lower total liver protein compared with the *ad libitum* and LPS-treated animals.

Total liver protein/kg body weight

When the total liver protein was expressed relative to body weight, the major finding was that animals fed the NP and the MP diets, but not animals fed the LP and the VLP diets, were able to increase the relative total protein after LPS treatment (Table 4.10).

In animals fed *ad libitum*, the total protein in liver/kg body weight was significantly higher in the NP group compared with the LP and the VLP groups, and it was significantly higher in the MP and the LP groups compared with the VLP group. After food restriction, no statistically significant difference was found in the relative hepatic protein between the groups. After LPS treatment, animals fed the NP and the MP groups had more protein in liver/kg body weight compared with the LP and the VLP groups, and it was higher in the LP group compared with the VLP group.

Comparing the LPS-treated rats, animals fed *ad libitum* and pair-fed controls, it can be seen that for each kg of body weight, LPS-treated animals fed the NP and the MP diet had a relatively more protein in liver compared with the corresponding *ad libitum* and pair-fed animals. In the LP and the VLP dietary groups, no significant differences were found due to treatments.

Table 4.10 Protein in liver and lung of rats fed diets of graded casein content, after starting treatments

Dietary groups g protein/kg diet	NP 180	MP 120	LP 80	VLP 60	two-way ANOVA p-value	F ratio
Liver protein (mg/g liver)						
ad libitum	158.9 ± 5.2 ^{a}	154.7 ± 4.4 ^{a}	146.3 ± 3.7 ^{a}	129.0 ± 4.8 ^{b}	D=0.000	F=8.3
pair-fed	162.8 ± 3.4 ^{a}	158.4 ± 6.1 ^{a}	160.0 ± 7.6 ^{a}	160.5 ± 4.2 ^{a}*	T=0.000	F=10.0
LPS	161.8 ± 3.8 ^{a}	149.1 ± 5.3 ^{ab}	136.4 ± 6.2 ^{b}♠	132.4 ± 6.2 ^{b}♠	I=0.052	F=2.2
Total liver protein (mg/total liver)						
ad libitum	716 ± 15 ^{a}	694 ± 46 ^{ab}	582 ± 26 ^{b}	467 ± 24 ^{c}	D=0.000	F=35.1
pair-fed	650 ± 41 ^{a}	554 ± 36 ^{a}*	538 ± 46 ^{ab}	429 ± 19 ^{b}	T=0.009	F=5.1
LPS	710 ± 29 ^{a}	684 ± 28 ^{a}♠	514 ± 22 ^{b}	437 ± 35 ^{b}	I=0.299	F=1.2
Relative liver protein (g/kg body weight)						
ad libitum	7.56 ± 0.15 ^{a}	7.1 ± 0.22 ^{ab}	6.7 ± 0.13 ^{b}	5.84 ± 0.17 ^{c}	D=0.000	F=21.5
pair-fed	6.62 ± 0.23 ^{a}*	6.43 ± 0.15 ^{a}*	6.76 ± 0.49 ^{a}	6.20 ± 0.14 ^{a}	T=0.000	F=16.9
LPS	8.50 ± 0.25 ^{a}♠*	8.25 ± 0.32 ^{a}♠*	7.1 ± 0.24 ^{b}	6.1 ± 0.20 ^{c}	I=0.001	F=4.5
Lung protein (mg/g lung)						
ad libitum	73.2 ± 2.0 ^{a}	77.5 ± 2.3 ^{a}	72.4 ± 3.5 ^{a}	73.4 ± 3.1 ^{a}	D=0.112	F=2.08
pair-fed	72.0 ± 2.5 ^{a}	79.6 ± 1.7 ^{a}	74.6 ± 3.1 ^{a}	77.6 ± 3.6 ^{a}	T=0.457	F=0.79
LPS	76.8 ± 1.7 ^{a}	78.3 ± 1.3 ^{a}	75.4 ± 2.6 ^{a}	74.2 ± 1.6 ^{a}	I=0.795	F=0.51
Total lung protein (mg/total lung)						
ad libitum	54.2 ± 1.2 ^{a}	60.2 ± 2.9 ^{a}	48.3 ± 1.9 ^{b}	45.1 ± 1.7 ^{b}	D=0.000	F=8.21
pair-fed	57.2 ± 3.4 ^{a}	51.6 ± 5.2 ^{ab}	47.5 ± 2.0 ^{b}	46.3 ± 4.4 ^{ab}	T=0.457	F=0.75
LPS	57.5 ± 3.5 ^{ab}	57.3 ± 1.5 ^{a}	51.7 ± 4.6 ^{ab}	47.2 ± 3.2 ^{b}	I=0.699	F=0.64
Relative lung protein (g/kg body weight)						
ad libitum	0.578 ± 0.001 ^{a}	0.617 ± 0.025 ^{a}	0.556 ± 0.028 ^{a}	0.568 ± 0.026 ^{a}	D=0.657	F=0.54
pair-fed	0.588 ± 0.022 ^{a}	0.597 ± 0.019 ^{a}	0.600 ± 0.030 ^{a}	0.665 ± 0.050 ^{a}	T=0.000	F=11.7
LPS	0.690 ± 0.036 ^{a} ♠*	0.691 ± 0.020 ^{a} ♠*	0.666 ± 0.025 ^{a} *	0.661 ± 0.030 ^{a} *	I=0.416	F=1.03

-Results are presented as mean ± standard error of the mean, n=6 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

Lung protein concentration

Animals were able to maintain lung protein concentration even under conditions of a severely protein deficiency diet. No significant difference in lung protein concentration was found due to diet or treatments between the groups (Table 4.10).

Total lung protein

The total lung protein, as in liver, was mainly influenced by dietary protein intake. In general, the total lung protein between animals of various dietary and treatment groups varied due to the difference in the size of lung (see Table 4.6 & Table 4.10).

A trend for a reduction in the total lung protein occurred when the protein content in the diet was decreased to 80 g or 60 g/kg diet. On the other hand, No significant difference was found due to treatments.

Total lung protein/kg body weight

No difference in the total protein in lung/kg body weight was found between the groups due to diet. Due to treatments, as in liver, the LPS-treated animals fed the NP and the MP diets were able to increase the amount of protein in lung/kg body weight compared with the corresponding pair-fed controls. The amount of protein in lung/kg body weight in LPS-treated animals fed the NP and MP diets were also significantly higher than the corresponding animals fed *ad libitum* (Table 4.10). Total lung protein/kg body weight in the LPS-treated animals fed the LP and the VLP diets was significantly higher than in the *ad libitum*-fed animals.

4.4.4 Serum concentrations of α-1 acid glycoprotein and albumin

α-1 acid glycoprotein

A greater than six fold increase in serum concentration of α-1 acid glycoprotein was observed in animals exposed to the inflammatory stimulus of LPS (Table 4.11).

In all dietary groups, α-1 acid glycoprotein increased dramatically in the LPS-treated animals compared with the corresponding *ad libitum* and pair-fed groups. However, the magnitude of the increase was lower in animals fed the very-low protein diet when compared to values from other dietary groups. The difference was not statistically significant. No significant difference was found in all dietary groups between the *ad libitum* and pair-fed controls.

Albumin

Albumin concentration was affected by feeding a diet low in protein, and by exposure to the inflammatory influence of LPS (Table 4.11).

Serum albumin concentration in the *ad libitum* and LPS-treated animals fed the low-protein diets (the LP and VLP diets) was lower than in animals fed the normal and moderate-protein diets (the NP and MP diets).

In all dietary groups, animals received LPS had a lower albumin concentration when compared with the corresponding pair-fed animals. Albumin concentration was also found to be lower in LPS-treated animals fed the NP, MP, and the VLP diets compared with the corresponding animals fed *ad libitum*.

Table 4.11 Serum α-1 acid glycoprotein and albumin of rats fed diets of graded casein content, after starting treatments

Dietary groups g protein/kg diet	NP 180	MP 120	LP 80	VLP 60	two-way ANOVA p-value F ratio
α-1 acid g lycoprotein (μg/ml)					
ad libitum	86 ± 5 [a]	100 ± 7 [a]	96 ± 5 [a]	98 ± 10 [a]	D=0.092 F=2.25
pair-fed	92 ± 6 [a]	107 ± 6 [a]	95 ± 7 [a]	104 ± 5 [a]	T=0.000 F=220.7
LPS	930 ± 96 [a]♠*	959 ± 65 [a]♠*	765 ± 99 [a]♠*	643 ± 132 [a]♠*	I=0.051 F=2.25
Albumin (mg/ml)					
ad libitum	35.2 ± 0.51[a]	34.8 ± 0.72 [a]	32.2 ± 0.69 [b]	32.1 ± 0.41 [b]	D=0.000 F=23.07
pair-fed	36.5 ± 0.91 [a]	36.0 ± 0.91 [ab]	35.0 ± 0.37 [b]*	32.7 ± 0.58 [c]	T=0.000 F=39.51
LPS	33.2 ± 0.55 [a]♠*	32.3 ± 0.34 [a]♠*	30.5 ± 0.53 [b]♠	29.7 ± 0.66 [b]♠*	I=0.663 F=0.68

-Results are presented as mean ± standard error of the mean, n=6 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

4.4.5 Inorganic sulphate excretion, and urinary nitrogenous end products of amino acid metabolism: Urea-N and ammonia-N excretion

Inorganic sulphate excretion, and inorganic sulphate excretion as a percentage of sulphur amino acid intake-average of two days before starting treatments

There was a progressive reduction in inorganic sulphate excretion (SO_4) as dietary protein concentration was decreased between the NP and the VLP groups. SO_4 excretion was slightly reduced in animals fed the VLP diet compared to values from animals fed the LP diet, however, the difference was not statistically significant (Table 4.12). When SO_4 excretion was expressed as a percentage of sulphur amino acid intake, no difference was found between the NP group and the MP group, and their excretion, as a percentage of sulphur amino acid intake, were significantly lower than the LP and the VLP groups. In the VLP group, the percentage of sulphur amino acid intake, which was excreted as SO_4, was significantly higher than the rest of the groups (Table 4.12). This may indicate that the efficiency of using sulphur amino acid, for anabolic purposes, in the MP group was similar to the NP group, and that this efficiency falls in the LP and the VLP groups.

Table 4.12 Inorganic sulphate excretion, and inorganic sulphate excretion as a percentage of sulphur amino acid intake (S.A.A.I.) of rats fed diets of graded casein content-average of two days, before starting treatments (μmol/day)

Dietary groups	NP	MP	LP	VLP	one-way ANOVA
g protein/kg diet	180	120	80	60	p-value F ratio
SO_4 excretion	185 ± 4.6^a	127 ± 6.5^b	110 ± 3.6^c	92 ± 8.4^c	p=0.000 F=44.3
(SO_4 excretion/ S. A.A.I.) x100	31.0 ± 0.74^a	33.2 ± 1.7^a	42.9 ± 1.2^b	51.0 ± 3.3^c	p=0.000 F=21.6

* Results are presented as mean ± standard error of the mean, n=12 per group. Means within each row having different letter superscripts following the number differ significantly.

Inorganic sulphate excretion after starting treatments

The results of SO_4 excretion can be found in Table 4.13. In animals fed *ad libitum*, there was a significant reduction in SO_4 excretion as dietary protein content was decreased between the NP group and the LP group. Further reduction occurred in the VLP group, but this was not statistically significant when compared to values from the LP group. The reduction in food intake, bought about by pair feeding, caused a significant reduction in SO_4 excretion in all dietary groups compared with the animals fed *ad libitum*. In the pair-fed controls and in LPS-treated animals, SO_4 excretion of the NP group was significantly higher than other dietary groups, and there was a trend for a reduction in SO_4 excretion between the MP group and the VLP group, but this trend was not statistically significant. Although the amount of sulphur amino acids eaten by the LPS-treated animals and pair-fed animals within the same dietary group was similar, the mean values was higher in the LPS-treated animals compared with the pair-fed animals. This difference was statistically significant in the NP and VLP groups. In the NP group the excretion of SO_4 in the LPS-treated animals was elevated to the same value of the animals fed *ad libitum*. In all other dietary groups (the MP, LP and VLP groups), the mean values of LPS-treated animals were lower than the observed in the corresponding animals fed *ad libitum*, the difference being statistically significant in the MP and VLP groups.

Table 4.13 Inorganic sulphate excretion of rats fed diets of graded casein content, after starting treatments (μmol/day)

Dietary groups	NP	MP	LP	VLP	two-way ANOVA	
g protein/kg diet	180	120	80	60	p-value	F ratio
ad libitum	157 ± 4.7^{a}	120 ± 3.8^{b}	101 ± 5.8^{c}	84 ± 4.1^{c}	D=0.000	F=56.1
pair-fed	106 ± 10.6^{a}*	62 ± 6.2^{b}*	50 ± 6.0^{b}*	44 ± 2.2^{b}*	T=0.000	F=34.4
LPS	176 ± 9.9^{a}♠	89 ± 8.8^{b}*	75 ± 12^{b}	64 ± 5.0^{b}♠*	I=0.045	F=2.4

-Results are presented as mean ± standard error of the mean, n=4 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

Ammonia-N and urea-N excretion-average of two days before starting treatments

Ammonia-N and urea-N excretion was influenced by dietary protein content. Table 4.14 shows that when protein content in the diet was decreased from 180 g to 60 g/kg diet, ammonia-N and urea-N excretion were reduced significantly. However, urea-N excretion was affected to a greater extent than that of ammonia-N excretion.

Table 4.14 Ammonia-N and urea-N excretion of rats fed diets of graded casein content -average of two days, before starting treatments (μmol/day)

Dietary groups	NP	MP	LP	VLP	one-way ANOVA	
g protein/kg diet	180	120	80	60	p-value	F ratio
Ammonia-N	457 ± 19^{a}	243 ± 20^{b}	115 ± 14^{c}	79 ± 8^{d}	p=0.000	F=115
Urea-N	7584 ± 306^{a}	3936 ± 224^{b}	1256 ± 74^{c}	850 ± 71^{d}	p=0.000	F=257

* Results are presented as mean ± standard error of the mean, n=12 per group. Means within each row having different letter superscripts following the number differ significantly.

Ammonia-N and urea-N excretion after starting treatments

a) Ammonia-N excretion after starting treatments

Dietary protein content and food restriction influenced ammonia-N excretion. When the LPS-treated animals are compared with the pair-fed animals, LPS treatment caused an increase in ammonia-N excretion only in the NP group (Table 4.15).

In the animals fed *ad libitum*, ammonia-N excretion was reduced significantly as the protein content in the diet was decreased. In the pair-fed and in LPS-treated animals, ammonia-N excretion of the NP group was significantly higher than the rest of the groups, it was significantly higher in the MP group compared with the LP and the VLP groups, and it was similar between the LP and the VLP groups. Comparing the LPS-treated animals with the pair-fed animals, it can be seen that ammonia-N excretion in the LPS-treated animals of the NP group was increased significantly compared with the corresponding pair-fed controls. In other groups, no difference in

the excretion of ammonia-N was found when the LPS-treated animals compared with the pair-fed controls.

b) Urea-N excretion after starting treatments

Urea-N excretion was influenced by dietary protein content, food restriction, and LPS treatment (Table 4.15).

In the animals fed *ad libitum*, urea-N excretion was reduced significantly as the protein concentration in the diet was decreased from 180 g to 60 g/kg diet. In the pair-fed animals, urea-N excretion decreased as the protein concentration in the diet was decreased from 180 g/kg diet to 60 g/kg diet. Furthermore, urea-N excretion in the pair-fed animals was significantly lower than in each corresponding *ad libitum* and LPS groups. In the LPS-treated animals, although the food intake was reduced compared with the *ad libitum*-fed animals, urea-N excretion was not decreased. Values followed the same trend as in the *ad libitum* groups in response to dietary protein concentration.

Table 4.15 Ammonia-N and urea-N excretion of rats fed diets of graded casein content, after starting treatments (μmol/day)

Dietary groups	NP	MP	LP	VLP	two-way ANOVA	
g protein/kg diet	180	120	80	60	p-value	F ratio
NH_3-N						
ad libitum	311 ± 16^{a}	150 ± 10^{b}	95 ± 7^{c}	62 ± 5^{d}	D=0.000	F=277
pair-fed	250 ± 16^{a}*	134 ± 9^{b}	63 ± 6^{c}*	44 ± 1^{c}*	T=0.001	F=8.2
LPS	342 ± 28^{a} ♠	128 ± 6^{b}	62 ± 6^{c}*	57 ± 4^{c}	I=0.003	F=4.1
Urea-N						
ad libitum	7356 ± 431^{a}	3942 ± 149^{b}	1561 ± 141^{c}	958 ± 76^{d}	D=0.000	F=427
pair-fed	4873 ± 251^{a}*	2870 ± 74^{b}*	974 ± 90^{c}*	453 ± 39^{d}*	T=0.000	F=34
LPS	7471 ± 482^{a}♠	3898 ± 207^{b}♠	1340 ± 117^{c}♠	827 ± 59^{d}♠	I=0.000	F=6.6

-Results are presented as mean ± standard error of the mean, n=4 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

4.4.6 Relationship between parameters measured in the study

The objective of processing data in this way is to develop a qualitative understanding of how sulphur amino acids, and other amino acids, are partitioned between various metabolic processes during growth, food restriction and inflammation. The parameters chosen for display in this way are major products of metabolism. In some cases they are end products (urinary urea-N, ammonia-N, inorganic sulphate), in other cases the item is subject to dynamic change in which the concentration is the result of the balance between the rates of synthesis and breakdown (liver protein) and inter organ flux (liver glutathione).

The ratio of glutathione to protein in liver

The ratio of glutathione to protein in liver, representing two major products of hepatic amino acid metabolism during growth and inflammation, can be seen in Table 4.16.

Dietary protein content, food intake and exposure to LPS exerted influences on the ratio. In the animals fed *ad libitum*, LPS-treated rats and pair-fed controls, the NP group had the highest ratio of GSH to protein in liver.

In the animals fed *ad libitum*, there was a significant reduction in the ratio of hepatic GSH to protein in rats fed the MP, LP and VLP diets compared with rats fed the NP diet. No significant difference in the ratio was found between the LP and the VLP groups. A similar phenomenon occurred during restricted feeding. In the pair-fed controls, the ratio was higher in animals fed the NP diet compared with animals fed the MP, LP, and the VLP diets. In the LPS-treated animals, the ratio was higher in animals fed the NP diet compared with other dietary groups, and it was higher in the MP group compared with the VLP group.

After food restriction, animals fed the NP and the MP diets, failed to maintain the ratio to the same level as in the *ad libitum* fed animals. There was 47% and 39% fall in the ratio of the pair-fed controls compared with the animals fed *ad libitum* in the NP and the MP groups, respectively. However, in the LP and VLP groups the ratios were reduced no further by food restriction. In animals exposed to the

inflammatory effects of LPS, the ratio of GSH to protein in liver was significantly higher than in the corresponding pair-fed controls in the NP, MP, and the LP groups, but not in animals fed the VLP diet.

Table 4.16 The ratio of hepatic glutathione to hepatic protein, and to inorganic sulphate excretion in urine of rats fed diets of graded casein content, after starting treatments

Dietary groups	NP	MP	LP	VLP	tow-way ANOVA	
g protein/kg diet	180	120	80	60	p-value	F ratio
GSH/protein (nmol/mg)						
ad libitum	34.8 ± 2.6 [a]	14.8 ± 1.0 [b]	10.8 ± 0.8 [c]	12.1 ± 1.5 [bc]	D=0.000	F=88.4
pair-fed	18.4 ± 2.2 [a]*	9.0 ± 1.0 [b]*	11.3 ± 0.6 [b]	11.1 ± 0.54 [b]	T=0.000	F=43.3
LPS	39.1 ± 2.3[a]♠	19.8 ± 1.0 [b]♠*	18.2 ± 2.0 [bc]♠*	14.3 ± 1.4[c]	I=0.000	F=7.79
GSH/SO_4 (nmol per liver /µmol per day)						
ad libitum	167 ± 16 [a]	82 ± 11[b]	68 ± 10 [b]	78 ± 1 [b]	D=0.001	F=7.43
pair-fed	121 ± 15 [a]	78 ± 6 [a]	130 ± 20 [a]	106 ± 9 [a]	T=0.004	F=6.40
LPS	146 ± 12[a]	153 ± 13 [a]♠*	143 ± 22[a b]*	92 ± 8 [b]	I=0.001	F=4.64

-Results are presented as mean ± standard error of the mean. For the ratio of hepatic glutathione to hepatic protein, n=6 per treatment group. For the ratio of hepatic glutathione to inorganic sulphate excretion, n=4 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

The ratio of total glutathione in liver to inorganic sulphate excreted in urine

The ratio of total glutathione in liver to inorganic sulphate excreted in urine is a qualitative index of processing of sulphur amino acids into the antioxidant defences of the animal and into major excretion products. The ratio can be seen in Table 4.16. Dietary protein intake only exerted a strong influence on the ratio in the absence of a restricted food intake and an inflammatory response.

In the animals fed *ad libitum*, the ratio of total hepatic GSH to SO_4 excretion was reduced significantly in the MP, LP and VLP groups compared with the NP group. In the pair-fed animals, no significant difference in the ratio was found between the groups. After LPS treatment, animals fed the MP and the LP diets, were able to increase the ratio compared with the *ad libitum* fed animals, to make values similar to LPS treated animals fed the NP diet. No significant increase in the ratio occurred after LPS treatment in rats fed the VLP diet.

Comparing the LPS-treated animals with the pair-fed controls, it can be seen that there was a trend for an increase in the ratio of total hepatic GSH to SO_4 excretion occurred in LPS-treated animals fed the NP, MP, and LP diets compared with the corresponding pair-fed controls, but this difference was statistically significant only in rats fed the MP diet.

The ratio of ammonia-N and urea-N excretion to N intake before starting treatments

Table 4.17 shows that the ratio of ammonia-N and urea-N excretion to N intake was reduced significantly as dietary protein content was decreased from 180 g to 80 g/kg diet. The ratio between urea-N and N intake was influenced to a much greater extent than that of ammonia-N by dietary N intake. No further reduction was observed when the protein concentration in the diet was decreased from 80 g to 60 g/kg diet.

Table 4.17 The ratio of ammonia-N and urea-N excretion to N intake of rats fed diets of graded casein content -average of two days, before starting treatments (mg/mg)

Dietary groups	NP	MP	LP	VLP	one-way ANOVA	
g protein/kg diet	180	120	80	60	p-value	F ratio
NH_3-N/N intake	0.017 ± 0.001^a	0.014 ± 0.001^b	0.010 ± 0.001^c	0.0104 ± 0.001^c	p=0.000	F=7.83
Urea-N/N intake	0.278 ± 0.009^a	0.225 ± 0.012^b	0.107 ± 0.006^c	0.105 ± 0.007^c	p=0.000	F=90.50

* Results are presented as mean ± standard error of the mean, n=12 per group. Means within each row having different letter superscripts following the number differ significantly.

The ratio of ammonia-N and urea-N excretion to N intake after starting treatments

a) The ratio of ammonia-N to N intake after starting treatments

The ratio of ammonia-N to N intake, while influenced by dietary protein concentration, was mostly unaffected by restricted feeding and exposure to the inflammatory influence of LPS (Table 4.18).

In the animals fed *ad libitum*, the ratio of ammonia-N excretion to N intake was reduced significantly as dietary protein content was decreased from 180 g to 80 g/kg diet. No further reduction occurred in the VLP group. In the pair-fed animals, the ratio was similar between the NP and the MP groups, and they were significantly higher than the LP and the VLP groups. No significant difference was found between the LP and the VLP groups. In the LPS-treated animals, there was a trend for a reduction in the ratio as dietary protein content was decreased from 180 g to 80 g/kg diet. This reduction was statistically significant when comparing the NP group with the LP group. No further reduction in the ratio occurred when the protein concentration in the diet was decreased from 80 g/kg diet to 60 g/kg diet. Comparing the LPS-treated animals with the animals fed *ad libitum*, it can be seen that LPS-treated animals fed the NP diet had a significantly higher ratio than in the *ad libitum* animals, Otherwise, no significant differences were found due to treatments.

b) The ratio of urea-N excretion to N intake after starting treatments

The ratio of urea-N excretion to N intake was affected by dietary protein content in the diet and by the inflammatory influence of exposure to LPS. Restriction in food intake had no effect upon the ratio (Table 4.18).

In the animals fed *ad libitum*, the ratio of urea-N excretion to N intake was significantly reduced as dietary protein content was decreased from 180 g to 80 g/kg diet. No further reduction in the ratio occurred when the dietary protein content fell to 60 g/kg diet. In the pair-fed animals, the ratio was similar between the NP and MP, and their excretion was higher than the LP and VLP groups. In the pair-fed animals, the ratio was lower in the VLP group than in other dietary groups. In the LPS-treated animals, the ratio was similar between the NP and MP groups, and their excretion was significantly higher than the LP and VLP groups. No significant difference was found between the LP and VLP groups.

Comparing the LPS-treated animals, *ad libitum* and pair-fed animals, it can be seen that in the NP, MP, and LP groups, the ratio was increased in the LPS-treated animals compared with the corresponding animals fed *ad libitum*. When comparing the LPS-treated animals with the pair-fed controls, it can be seen that in the NP and VLP groups, the values were higher in the LPS-treated animals compared with the corresponding pair-fed controls. The significant increase in the ratio which was found in the NP and the VLP group after LPS treatment compared with the pair-fed animals, was numerically larger in the NP group. 0.185 mg of extra urea-N/mg of N eaten, was excreted in urine of animals fed the NP diet, compared with only 0.06 mg of extra urea-N/ mg of N eaten, was excreted in urine of animals fed the VLP diet.

Table 4.18 The ratio of ammonia-N and urea-N excretion to N intake of rats fed diets of graded casein content, after starting treatments (mg/mg)

Dietary groups	NP	MP	LP	VLP	tow-way ANOVA	
g protein/kg diet	180	120	80	60	p-value	F ratio
NH_3-N/N intake						
ad libitum	0.013 ± 0.001^{a}	0.010 ± 0.0001^{b}	0.008 ± 0.0004^{c}	0.007 ± 0.001^{c}	D=0.000	F=16.1
pair-fed	0.014 ± 0.001^{a}	0.016 ± 0.003^{a}	0.010 ± 0.001^{b}	0.008 ± 0.0004^{b}	T=0.015	F= 4.7
LPS	0.018 ± 0.002^{a}*	0.015 ± 0.003^{a}	0.008 ± 0.001^{b}	0.009 ± 0.001^{b}	I=0.123	F= 1.8
Urea-N/N intake						
ad libitum	0.280 ± 0.006^{a}	0.252 ± 0.009^{b}	0.138 ± 0.005^{c}	0.110 ± 0.012^{c}	D=0.000	F=75.8
pair-fed	0.270 ± 0.019^{a}	0.350 ± 0.056^{a}	0.161 ± 0.015^{b}	0.080 ± 0.006^{c}	T= 0.000	F=28.0
LPS	0.455 ± 0.045^{a}♠*	0.503 ± 0.037^{a}*	0.181 ± 0.010^{b}*	0.140 ± 0.015^{b}♠	I= 0.001	F= 5.3

-Results are presented as mean ± standard error of the mean, n=4 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

The ratio of inorganic sulphate excretion to ammonia-N and urea-N excretion- average of two days before starting treatments

Since all amino acids donate N to ammonia and urea formation but only sulphur amino acids contribute to sulphate excretion, the ratios indicate whether sulphur amino acid metabolism is distinctive in it response, compared to all other amino acids, to changes in dietary protein intake, food restriction and inflammation.

a) The ratio of inorganic sulphate excretion to ammonia-N before starting treatments

The ratio of SO_4 excretion to NH_3-N excretion in urine was significantly increased as the protein concentration in the diet was decreased between the NP and the LP group. In the VLP group, the magnitude of the ratio was higher than the LP group, but this difference was not statistically significant (Table 4.19). The ratio was increased by 34%, 174% and 233% respectively in the MP, LP, VLP groups compared with the NP group.

b) The ratio of inorganic sulphate excretion to urea-N excretion before starting treatments

There was a significant increase in the ratio of SO_4 excretion to urea-N excretion as the protein content in the diet was decreased (Table 4.19). The ratio was increased by 32%, 248% and 360% respectively in the MP, LP, VLP compared with the NP group. These results clearly indicate the relatively greater inability of animals fed the LP and the VLP diets to utilise sulphur amino acids than to use other amino acids efficiently for biosynthetic processes.

Table 4.19 The ratio of inorganic sulphate excretion to ammonia-N and urea-N excretion of rats fed diets of graded casein content-average of two days before starting treatments (μmol/μmol)

Dietary groups	NP	MP	LP	VLP	one-way ANOVA	
g protein/kg diet	180	120	80	60	p-value	F ratio
SO_4/NH_3-N	0.41 ± 0.01^a	0.55 ± 0.04^b	1.12 ± 0.12^c	1.36 ± 0.22^c	p=0.000	F=13
SO_4/ urea-N	0.025 ± 0.001^a	0.033 ± 0.015^b	0.087 ± 0.003^c	0.115 ± 0.012^d	P=0.000	F= 45

* Results are presented as mean ± standard error of the mean, n=12 per group. Means within each row having different letter superscripts following the number differ significantly.

The ratio of SO_4 excretion to ammonia-N and urea-N excretion of rats fed diets of graded casein content after starting treatments

a) The ratio of inorganic sulphate excretion to ammonia-N after starting treatments

The results of this ratio can be seen in (Table 4.20). In the animals fed *ad libitum*, there was a trend for an increase in the ratio of SO_4 to NH_3-N excretion as the protein concentration in the diet was decreased between the NP and the VLP groups. This trend was statistically significant when comparing animals fed the NP diet with animals fed the LP and VLP diets, and when comparing animals fed the MP diet with animals fed the VLP diet. Neither a reduction in food intake nor the inflammatory response of LPS influenced the pattern of response to dietary protein content. In the pair fed animals, it was significantly lower in the NP and MP groups compared with the VLP group. In the LPS-treated animals, the ratio was significantly lower in the NP group compared with other groups, and it was significantly lower in the MP group compared with the VLP group. Due to treatments, in all the groups, the mean values was higher in the LPS-treated animals compared with the corresponding pair-fed controls. The difference was statistically significant in the MP group.

b) The ratio of inorganic sulphate excretion to urea-N excretion after starting treatments

The ratio of SO_4 to urea-N excretion was only affected by dietary protein content. Neither LPS treatment, nor food restriction caused alteration in this ratio (Table 4.20).

In the animals fed *ad libitum*, the ratio of inorganic sulphate excretion to urea-N excretion increased significantly as the protein content of diet was decreased. In the pair-fed animals, the ratio was similar between the NP group and the MP group, and their ratios were significantly lower than in the LP and VLP groups. The ratio was significantly higher in the VLP group compared with the LP group. In the LPS-

treated animals, the ratio was similar between the NP group and the MP group, and their ratios were significantly lower than in the LP and VLP groups. There was a trend for an increase in the ratio found in the VLP group compared with the LP group, but this increase in the ratio was not statistically significant. From this data, the conclusion that can be made is that animals fed the low-protein diets (the LP and VLP diets) whether fed *ad libitum*, under food restriction or during inflammation, are unable to reduce the amount of sulphur amino acids excreted as SO_4.

Table 4.20 The ratio of inorganic sulphate excretion to ammonia-N and urea-N excretion of rats fed diets of graded casein content, after starting treatments (μmol/μmol)

Dietary groups	NP	MP	LP	VLP	tow-way ANOVA	
g protein/kg diet	180	120	80	60	p-value	F ratio
SO_4/NH_3-N						
ad libitum	0.51 ± 0.02^{a}	0.75 ± 0.10^{ab}	1.07 ± 0.08^{bc}	1.39 ± 0.16^{c}	D=0.000	F=19.4
pair-fed	0.43 ± 0.04^{a}	0.46 ± 0.05^{a}	0.84 ± 0.15^{ab}	1.00 ± 0.062^{b}	T=0.021	F=4.31
LPS	0.52 ± 0.02^{a}	0.69 ± 0.04^{b} ♠	1.27 ± 0.27^{bc}	1.14 ± 0.11^{c}	I=0.615	F=0.75
SO_4/urea-N						
ad libitum	0.022 ± 0.001^{a}	0.031 ± 0.002^{b}	0.066 ± 0.004^{c}	0.089 ± 0.006^{d}	D= 0.000	F=128
pair-fed	0.022 ± 0.002^{a}	0.021 ± 0.002^{a}	0.054 ± 0.009^{b}	0.099 ± 0.009^{c}	T= 0.140	F=2.07
LPS	0.024 ± 0.001^{a}	0.023 ± 0.002^{a}	0.056 ± 0.008^{b}	0.078 ± 0.004^{b}	I= 0.150	F=1.70

-Results are presented as mean ± standard error of the mean, n=4 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

4.4.7 Number of neutrophils in lung

Feeding a diet deficient in protein reduced the number of neutrophils in lung of the *ad libitum* and pair-fed animals (Table 4.21). When an inflammatory stress was applied a different response occurred. Diets with the low-protein content significantly increased the number of neutrophils in the LPS-treated animals compared with the corresponding animals fed *ad libitum* and pair-fed controls. In the LPS-treated animals, no significant difference was found in the number of neutrophils. However, a trend for an increase in neutrophilic cells found in animals fed the low-protein diets (the LP and VLP diets) compared with animals fed the normal protein and moderate protein diets (the NP and MP diets).

Table 4.21 Number of neutrophils in lung of animals fed graded levels of casein (number of neutrophils in lung section/number of intersection points occupied by lung section) × 100

Dietary groups	NP	MP	LP	VLP	tow-way ANOVA	
g protein/kg diet	180	120	80	60	p-value	F ratio
ad libitum	15.0 ± 0.6 ^{a}	14.6 ± 0.7 ^{ab}	12.3 ± 0.6 ^{bc}	11.4 ± 0.7 ^{c}	D=0.038	F=3.11
pair-fed	14.5 ± 0.5 ^{a}	14.4 ± 0.8 ^{a}	11.3 ± 0.4 ^{b}	10.6 ± 0.4 ^{b}	T=0.000	F=31.5
LPS	16.1 ± 1.2^{a}	15.8 ± 0.73 ^{a}	17.2 ± 1.1 ^{a} ♠ *	18.9 ± 1.4^{a} ♠ *	I=0.001	F=5.15

-Results are presented as mean ± standard error of the mean, n=4 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

4.5 Discussion

Most of the metabolic activities measured in this study are actually centred in the liver, for example:

1) GSH in lung, spleen, and thymus depends mostly in GSH synthesised in the liver.

2) The number of neutrophils in lung may be indirectly affected by GSH in liver; e.g. liver GSH ⟶ lung GSH ⟶ number of neutrophils in lung.

3) The serum concentrations of the positive acute phase protein, α-1 acid glycoprotein, and the negative acute phase protein, albumin, depend on the capacity of the liver to synthesise these compounds.

4) The liver is the only organ in rats which has a full capacity to recycle methionine, and has all the enzymes required to synthesised cysteine from methionine via the transmethylation-transsulphuration pathway (Finkelstein, 1990). Furthermore, nitrogenous end products of amino acids metabolism (SO_4, urea, and ammonia) are mostly produced in liver.

Clearly the diets employed in the study had major effects on growth and organ weights in the various groups. Reducing the protein content in the diet significantly reduced the averaged daily weight gain of animals (Table 4.3). To minimise differences due to body weight in interpreting the data, the weights of organs are expressed as a proportion of body weight (Table 4.6). In the animals fed *ad libitum*, the relative weights of liver, lung, and thymus were similar between the groups. These results indicate that the reduction in the total weights of these organs, found after feeding the low-protein diets, was in proportion to the reduction in body weight. However, this was not the case in spleen. In the *ad libitum*-fed animals, the relative spleen weight was significantly decreased in the low-protein groups (the LP and VLP groups) compared with the normal and moderate-protein groups (the NP and MP groups). As in the *ad libitum*-fed animals, the relative spleen weight, after pair feeding and after giving LPS, decreased in animals fed the LP and VLP diets compared with animals fed the NP and MP diets. However, this reduction reached statistical significance in the LPS-treated animals, when comparing the VLP group

with the NP and MP groups. After LPS treatment, all animals had raised relative liver weight compared with the corresponding pair-fed controls. Certain aspects of acute phase response (e.g. gluconeogenesis & acute phase protein synthesis) may account for the increase in the relative liver weight in animals exposed to the inflammatory response of LPS. The increase in the relative liver weight, after LPS treatment in animals fed the low-protein diets, does not necessarily reflect the changes in protein content after exposure to the inflammatory response of LPS. After administering turpentine, as a model of injury, Jennings & Elia (1996) demonstrated that the changes in organ weights do not reflect the changes in tissue composition. For example, turpentine injection caused a significant change in the protein content of muscle and heart but not in their mass. To some extent, the presence of an inflammatory response partly reversed the fall in the relative spleen weight caused by reducing the protein content in the diet. This may be due to the amino acid released from endogenous sources under the action of LPS.

The data suggests that SO_4 excretion relates closely to sulphur amino acid intake, as sulphur amino acid intake increased, SO_4 excretion also increased (Table 4.3 & Table 4.12). However, sulphur amino acid intake is not the only factor which can influenced the excretion of SO_4. The other factor, which should be involved in this relationship, is the efficiency of using sulphur amino acid for anabolic purposes. When SO_4 excretion, was expressed as a percentage of sulphur amino acid intake (Table 4.12), animals fed the low-protein diets show a significantly increased ratio, particularly in animals fed the VLP diet, indicating the poor efficiency of using sulphur amino acid for anabolic purposes. This poor efficiency of using sulphur amino acid may be because the amount of sulphur amino acids consumed by the LP and the VLP animals, and present at the site of protein and glutathione synthesis, was inadequate to be used efficiently for synthesising these compounds, or that due to other amino acids being present in limiting amounts in the LP and the VLP groups, synthesis of these compounds is impaired and unutilised sulphur amino acids are catabolised to SO_4. The data in the present study disagrees with the in vitro study conducted by Stipanuk *et al.* (1992). They found that the percentage of cysteine

converted to sulphate was unaffected by cysteine availability. The percentage of cysteine converted to sulphate was similar between rat hepatocyes incubated with 0.05 mmol/l or with 1.0 mmol/l [^{35}S] cysteine. The in vitro study, of course, did not take into account the usage of cysteine for whole body protein synthesis (e.g. growth).

Taurine production, which is not measured in the present study, is one of the major end product of sulphur amino acid catabolism (Finkelstein, 1990). Bella & Stipanuk (1995) have shown that urinary taurine excretion, as a percentage of total urinary taurine and sulphate excretion, was significantly greater for rats fed a high-protein diet (6.4%) than in rats fed a low-protein diet (1.7%).

The ratio of SO_4 to urea-N excretion, before starting treatments (Table 4.19), was significantly increased as dietary protein content in the diet was decreased, supporting the latter possibility. The ratio was increased by 32%, 248% and 360% respectively in the MP, LP and VLP groups compared with the NP group. The situation when an inflammatory stress was applied was somewhat similar (Table 4.20). Neither LPS treatment nor restriction in food intake caused a significant alteration on the ratio.

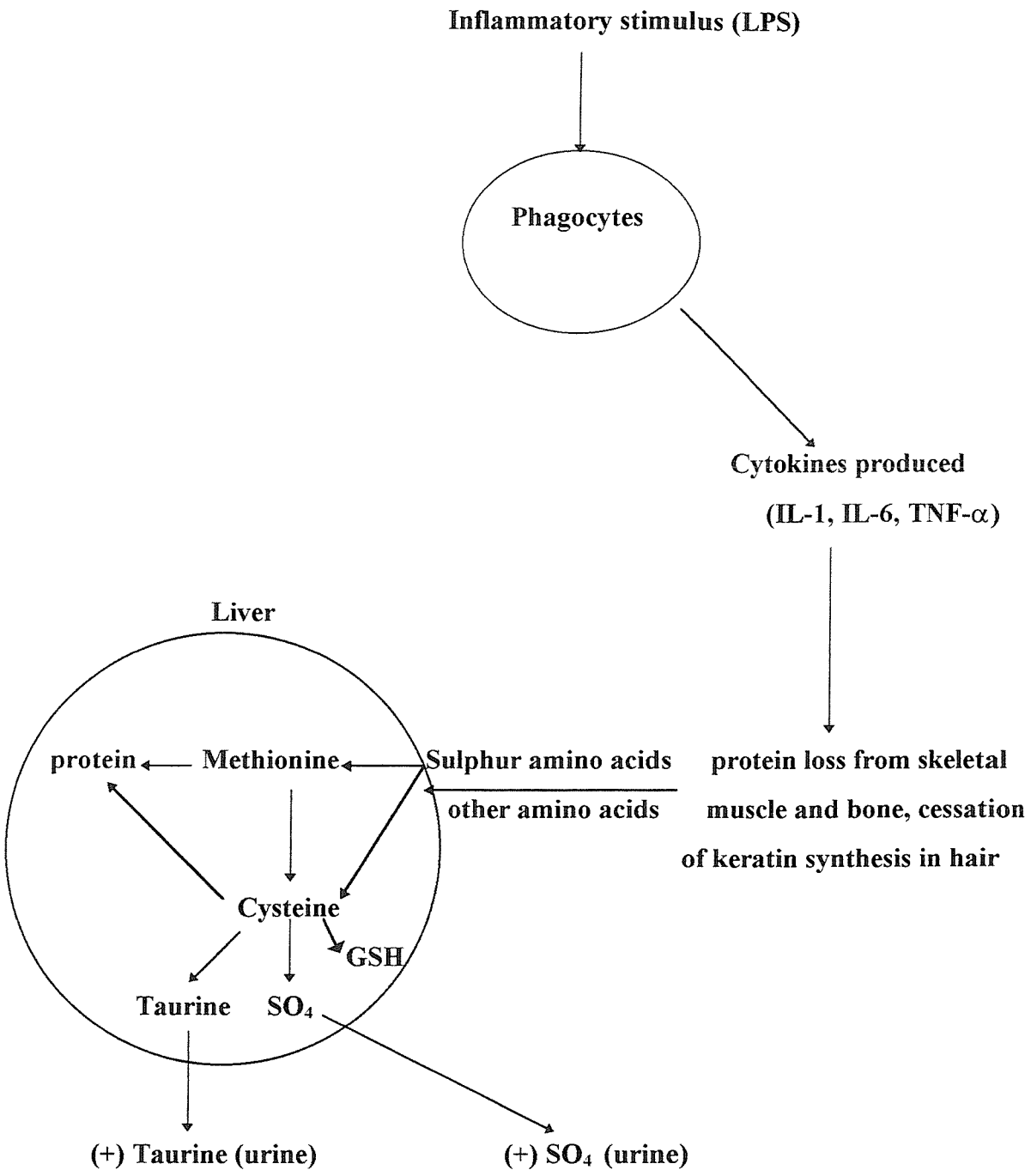
In the present study, the mean values of SO_4 excretion in the LPS-treated animals was higher than in the pair-fed animals, despite the similarity in the amount of food eaten (Table 4.13). This could be explained by the action of cytokines produced during the inflammatory response to LPS.

Exposure to lipopolysaccharides from the cell wall of bacteria can activate macrophages leading to the production of cytokines (Dinarello, 1984; Dinarello, 1986; Miossec *et al.* 1986; Grimble, 1990). Cytokines, such as TNF-α and IL-1, cause loss of muscle protein, which could be due to both increased proteolysis and decreased synthesis (Barcos *et al.* 1983; Charter & Grimble, 1989). When endogenous production of IL-1 is stimulated by LPS, less sulphur amino acids are used for hair synthesis, keratin synthesis, and protein loss from muscle and bone may occur (Wan *et al.* 1985). This could explain the increase in SO_4 excretion of the

LPS-treated animals compared with the pair-fed controls, despite a similarity in food intake (see Figure 4-2).

It was interesting to note that the ratio of SO_4 to urea-N in all dietary groups, and the ratio of SO_4 to NH_3-N in the NP, LP, and VLP groups were not changed during inflammation, despite the additional amino acid supply which came mainly from muscle protein breakdown. Thus, paradoxically, endogenous sources appear to be unable to supply the amino acids to the liver, and other tissues for synthesis of the molecules associated with inflammation, and do not appear to improve the underlying inefficiency for utilising sulphur amino acids which has been caused by a reduced casein intake. This phenomenon is evidence of the mismatch between amino acid supply and demands during inflammation, which was hypothesised by Reeds *et al.* (1994), and is made worse by a low-protein intake.

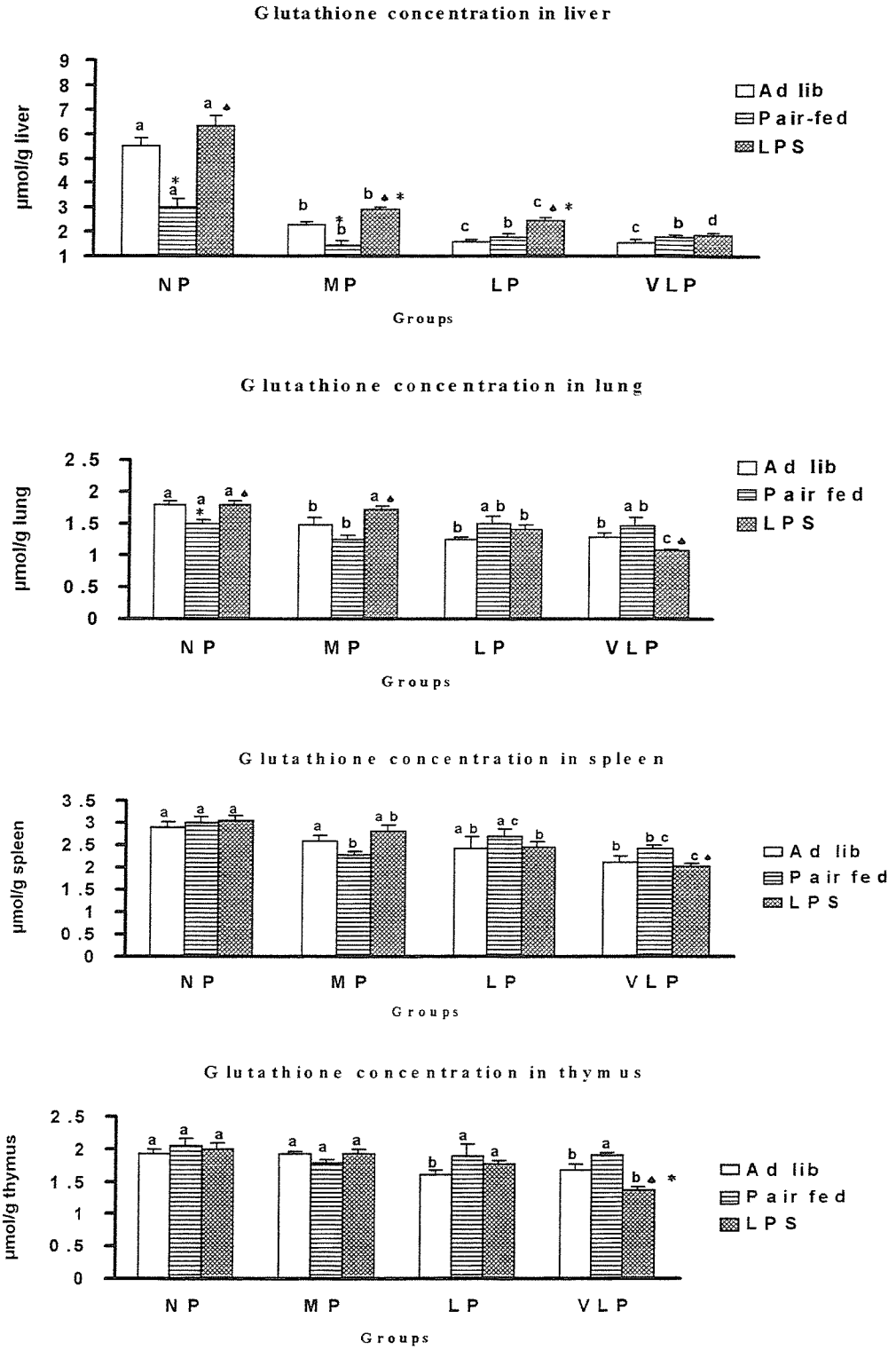
Figure 4-2 The effect of cytokines on sulphur amino acid metabolism



Biochemical information from the present project and the results of previous studies suggest that the low dietary protein intake in the present study provides insufficient sulphur amino acids to support the synthesis of glutathione and protein.

GSH is synthesised from three amino acids namely, glycine, glutamate and cysteine, of which cysteine is the limiting amino acid for synthesis (Meister *et al.* 1986). It can be seen from Table 4.7 & Figure 4-3 that in the animals fed *ad libitum*, hepatic GSH concentration was severely affected when the protein concentration in the diet was decreased from 180 g to 80 g protein/kg diet. No further reduction in hepatic GSH concentration was found when the protein content in the diet was decreased from 80 g to 60 g/kg diet. The major source of cysteine in the present experiment is L-methionine (see Table 4.2). Methionine can be converted to cysteine via the transmethylation-transsulphuration pathway. Since cysteine, and methionine which can be converted to cysteine, are the most limiting for GSH synthesis, the reduction in GSH concentration was mainly due to the decrease concentration of sulphur amino acids in the diet, as dietary protein content was decreased.

Figure 4-3 Glutathione concentration in liver, lung, spleen, and thymus



Results are mean ± SE, n=6 per treatment group. Means within each treatment group having different letter superscripts differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

When rats were fed a low-protein diet supplemented with sulphur amino acids, to be isosulphurous to the amount present in a normal-protein diet, hepatic GSH concentration was increased to the level found in animals fed the normal-protein diet (Bauman *et al.* 1988a), indicating that sulphur amino acids, but not protein concentration are limiting for GSH synthesis. In the present study, in the LPS-treated animals, hepatic GSH concentration was severely affected as dietary protein content in the diet was decreased. Thus the endogenous supply of amino acids, as a result of muscle protein breakdown under the action of cytokines, plus the low amount of amino acids, consumed by animals in the MP, LP, and VLP groups, were unable to enhance GSH concentration to the same level as in the NP group. In the pair-fed controls, given the NP and MP diets, hepatic GSH was severely affected both in concentration and in proportion to protein by further restriction of sulphur amino acid intake by an imposed reduction in food intake. Amino acids, which are released from increased muscle protein breakdown under these circumstances, are unable to compensate for the dietary lack in the NP and MP groups. Bauman *et al.* (1988b) and Morand *et al.* (1997) have shown that under food-restricted conditions, hepatic GSH concentrations in animals fed a normal-protein diet were affected to a greater extent by restriction in food intake than in animals fed a low-protein diet. When, however, muscle protein breakdown was accelerated during inflammation, the response was somewhat different. Comparing the LPS-treated animals with the pair-fed controls, it can be seen that hepatic GSH concentration was significantly increased after LPS treatment in the NP, MP, and LP groups, but not in the VLP group. Yet interestingly, despite the increased utilisation of sulphur amino acids for GSH synthesis in most groups, which this observation suggests, the SO_4 to urea-N or SO_4 to NH_3-N ratios were not different between the LPS-treated rats and the pair-fed animals.

Figure 4-3 shows that GSH concentration, in all four tissues in which it was measured (liver, lung, spleen and thymus), responds to a similar way to a reduction in protein intake. In lung, spleen, and thymus, GSH concentration of animals fed the VLP diet and exposed to LPS was significantly lower than in the corresponding pair-

fed controls. This may indicate the higher utilisation of GSH after LPS treatment, due to increased oxidant stress, accompanied by a low capacity for synthesising GSH in animals fed this severely protein-restricted diet. Lung and spleen are incapable of synthesising cysteine from methionine because these organs lacked cystathionine synthase and depend mostly on plasma cysteine and GSH for the cysteine supply (Finkelstein, 1990). Thus changes in lung and spleen GSH will depend upon uptake of GSH synthesised elsewhere, mostly by the liver.

The increase in hepatic GSH concentration in the LPS-treated animals fed the NP, MP and LP diets and the increase in lung GSH concentration in animals given LPS and fed the NP and MP diets, compared with the corresponding pair-fed controls, may be explained by both the effect of cytokines on the metabolism of amino acids (see Figure 4-2), and by the dietary intake of sulphur amino acids.

A previous study showed that the activity of γ-glutamyl cysteine synthetase was not affected by restriction in food intake (Tateishi *et al.* 1974). Furthermore, previous studies carried out in our laboratory by Langley *et al.* (1994) and Alhamdan & Grimble (1997) have shown that γ-glutamyl cysteine synthetase was not affected by restriction in food intake, or endotoxin treatment. From these studies, it can be concluded that the flow of substrate through the biosynthetic pathway of GSH, but not γ-glutamyl cysteine synthetase activity is rate limiting for glutathione synthesis in the present study.

Plasma GSH cannot be transported into the lung, spleen, and thymus as such. However, first GSH is broken down by an extracellular membrane enzyme, γ-glutamyl transferase, to γ-glutamyl amino acid and cysteinyl glycine (Allison & Meister, 1981), and then re-synthesised back to GSH intracellularly (Meister, 1989) (see Figure 1-11, chapter 1).

The magnitude of the reduction in GSH concentration between the NP and the VLP diet was much greater in liver than in other organs. The liver is the major site for the synthesis of plasma GSH (Lauterburg *et al.* 1984), and this may suggest that transport to lung, spleen and thymus is continued despite the low hepatic GSH

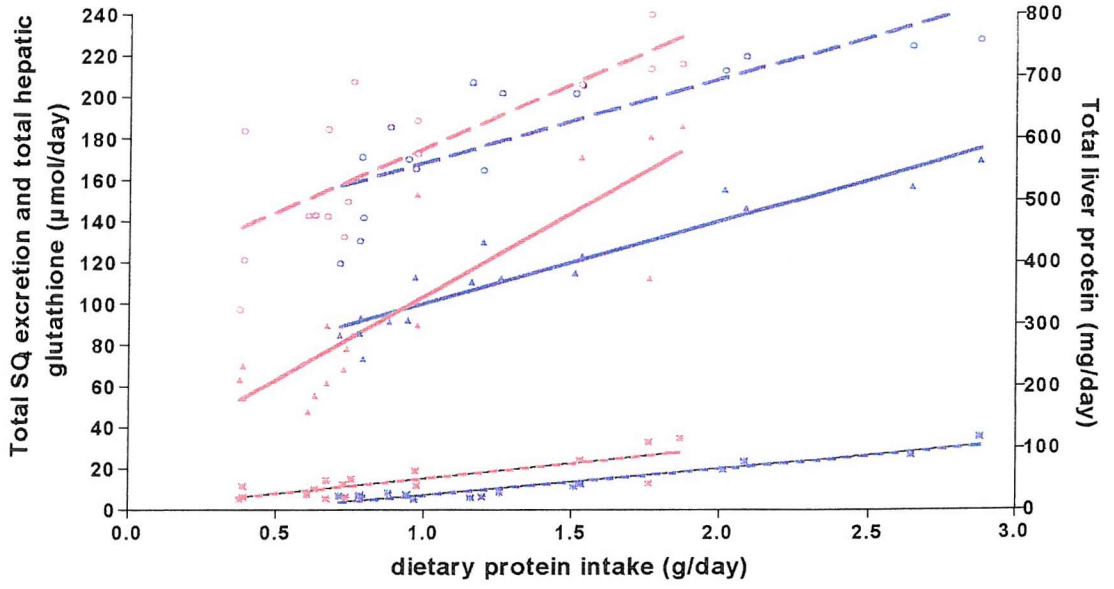
found in the low-protein dietary groups. Furthermore, the data indicates that GSH synthesis in liver is more sensitive to diet and inflammation than in other organs.

The liver total protein/kg body weight (Table 4.10) was increased significantly in the LPS-treated animals compared with the pair-fed animals in the NP and MP groups. The significant increase in the relative liver protein content in the LPS-treated rats fed the NP and MP diets was not observed in animals fed the low and very low-protein diets . In the LPS-treated animals, the result of relative liver protein was consistent with the result of serum α-1 acid glycoprotein (Table 4.11). In the LPS-treated animals, the serum concentration of this major acute phase protein increased to a lesser extent in animals fed the low and very low-protein diets compared with animals fed the normal-protein diet. The relative increase of α-1 acid glycoprotein after endotoxin treatment was 911%, 796%, 705%, and 518% compared with the corresponding pair-fed controls in rats fed the NP, MP, LP and VLP diets, respectively. This may suggest that animals fed diet severely deficient in protein were incapable of responding efficiently to the cytokines produced during the acute phase response. While in *ad libitum* and in pair-fed animals, α-1 acid glycoprotein was not affected by dietary protein intake, serum albumin concentration was affected by reducing the protein content in all treatment groups (Table 4.11). Furthermore, after exposure to LPS, albumin concentration was reduced in all dietary groups compared with the corresponding pair-fed controls.

Now, if we look at the results in a different way, and see the pattern of response of main products of sulphur amino acid metabolism (GSH and protein in liver, and SO_4 excretion) in animals receiving amino acid supplies from diet and normal protein turnover (animals fed *ad libitum*), or from both low-dietary intake, and from enhanced endogenous sources during inflammation (LPS-treated animals), and correlate these parameters with dietary protein intake, differing patterns of response emerge (Figure 4-4). Expressing data in this way gives a qualitative rather than a quantitative view of the relative responses of GSH, SO_4, and protein to amino acid supply, since protein turnover was not assessed in the present study. In the

figure, the data lines for the LPS-treated animals would shift to the right if total amino acid availability could be plotted on the X-axis.

Figure 4-4 Pattern of response of main products of sulphur amino acids in animals receiving amino acids supply from diet and normal protein catabolism and in animals receiving amino acids supply from diet and from endogenous sources (e.g. muscle protein catabolism)



n=16 for *ad libitum* animals.
n=16 for LPS-treated animals.

Inorganic sulphate excretion (Ad lib)
r 0.939; R squared 0.882; p<0.0001

Inorganic sulphate excretion (LPS)
r 0.860; R squared 0.750; p<0.0001

Total hepatic glutathione (Ad lib)
r 0.963; R squared 0.927; p<0.0001

Total hepatic glutathione (LPS)
r 0.825; R squared 0.681; p<0.0001

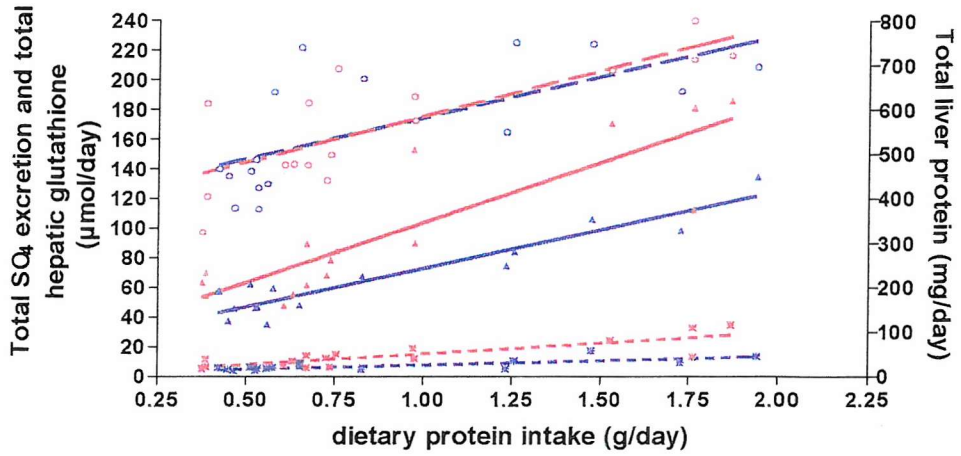
Total liver protein (Ad lib)
r 0.816; R squared 0.666; p<0.0001

Total liver protein (LPS)
r 0.783; R squared 0.628; p<0.0002

The results showed that the total hepatic GSH and protein, and urinary SO_4 excretion were positively correlated with dietary protein intake, regardless of whether animals were fed *ad libitum* or given endotoxin. However the magnitude of the correlation was stronger in the animals fed *ad libitum* than in the LPS-treated

animals. It can also be conclude from Figure 4-4, that at each amount of dietary protein intake, hepatic GSH in the LPS-treated animals was relatively higher than in the *ad libitum*-fed animals, indicating the importance of both, dietary protein intake, and endogenous protein catabolism, in supporting GSH synthesis in liver. However, for SO_4 excretion and total protein in liver, the sources of amino acid supply influence the slope of the line. When amino acids were derived from a mixture of diet and enhanced endogenous sources during inflammation, the slope of the line is steeper than when in the absence of inflammation. Furthermore, the lines of best fit for the SO_4 excretion and total liver protein of the *ad libitum* and LPS-treated animals are widely divergent at high protein intakes and intersect at low-protein intakes. This phenomenon did not occur for hepatic GSH. In Figure 4-5, the relationship between dietary sulphur amino acid intake and urinary SO_4 excretion, and total hepatic protein and glutathione is shown for the pair-fed and LPS-treated animals. This comparison permits an examination of whether amino acids derived from the diet are treated in a similar or different manner to those arising from endogenous sources, as dietary intake is altered. In both cases dietary intake was almost identical in each LPS injected rat and its pair-fed control. The flow of substrate from endogenous sources will be enhanced in the LPS animals. If this additional amino acid supply, released during inflammation, is processed in a similar way to that in the non-inflamed animal then the lines of best fit will be parallel. This is clearly not the case. Figure 4-5 shows that total GSH and protein in liver, and SO_4 excretion were positively correlated with dietary protein intake. However, hepatic GSH in the LPS-treated animals became relatively higher than in the pair-fed controls as the lines become more divergent at high protein intakes. The lines of best fit for hepatic protein and SO_4 data for the pair-fed and LPS-treated animals are also widely divergent at high protein intakes, but only that for SO_4 does not intersect at low-protein intakes.

Figure 4-5 Comparison of the pattern of response of main products of sulphur amino acids between pair-fed and LPS-treated animals



n=16 for LPS-treated animals.
n=16 for pair-fed animals.

Inorganic sulphate excretion (pair-fed)
r 0.941; R squared 0.886; P<0.0001

Inorganic sulphate excretion (LPS)
r 0.860; R squared 0.750; P<0.0001

Total hepatic glutathione (Pair-fed)
r 0.776;R squared 0.602; p<0.0004

Total hepatic glutathione (LPS)
r 0.825; R squared 0.681; P< 0.0001

Total liver protein (Pair-fed)
r 0.676; R squared 0.458; p<0.0040

Total liver protein (LPS)
r 0.783; R squared 0.628; p<0.0002

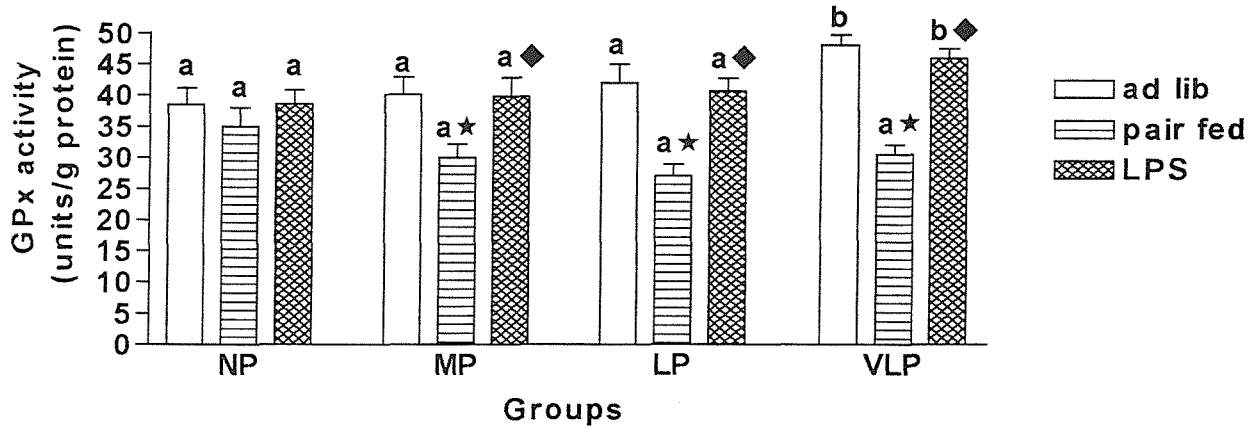
In general, it could be concluded that when amino acids come from endogenous sources during inflammation, and dietary protein intake was not severely restricted, relatively more sulphur amino acids metabolised to SO_4 and to liver protein than when amino acids come more predominantly from dietary intake. However, when dietary protein intake was severely restricted and amino acids come to a greater extent from endogenous sources during inflammation, relatively less sulphur amino acids metabolised to SO_4 and to liver protein than when amino acids come to a greater extent from dietary intake.

While examination of GSH content of organs gives an index of the synthesis and catabolism of this compound, observation of the changes in GSH peroxidase and GSSG reductase, give an indication of the rate of recycling of glutathione between its reduced (GSH) and oxidised (GSSG) forms. Hepatic GSH peroxidase activity

(see Table 4.8 & Figure 4-6) increased significantly in the LPS-treated animals fed the MP, LP and VLP diets when compared with the pair-fed animals, indicating the increase utilisation of the reduced form of glutathione during the inflammatory response.

In general, when dietary protein intake fell to 60 g/kg diet, activities of both hepatic GSH peroxidase (Table 4.8 & Figure 4-6) and GSSG reductase (Table 4.9) increased both in *ad libitum* and in LPS-treated animals, and fell when food intake was restricted. The former effect might increase the capacity of the organ for recycling the molecule between oxidised and reduced forms, and the latter effect might decrease the capacity. However, the much greater activity of GSSG reductase than GSH peroxidase (>1000 ×) would ensure a high relative GSH:GSSG ratio in tissue, provided that the necessary substrate was available.

Figure 4-6 Glutathione peroxidase activity in liver



Results are mean ± SE, n=4 per treatment group. Means within each treatment group having different letter superscripts differ significantly. ★ indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ◆ indicates a significant difference between pair-fed and LPS-treated groups.

Glutathione is not only important in conditions where oxidant molecules are increased, but also under normal conditions. For example, glutathione deficiency leads to damage of various tissues in mice and rats in the absence of oxidant agents (Meister, 1991). Mitochondria produce hydrogen peroxide from oxygen under normal physiological conditions (Boveris & Chance, 1973). Mitochondria depend

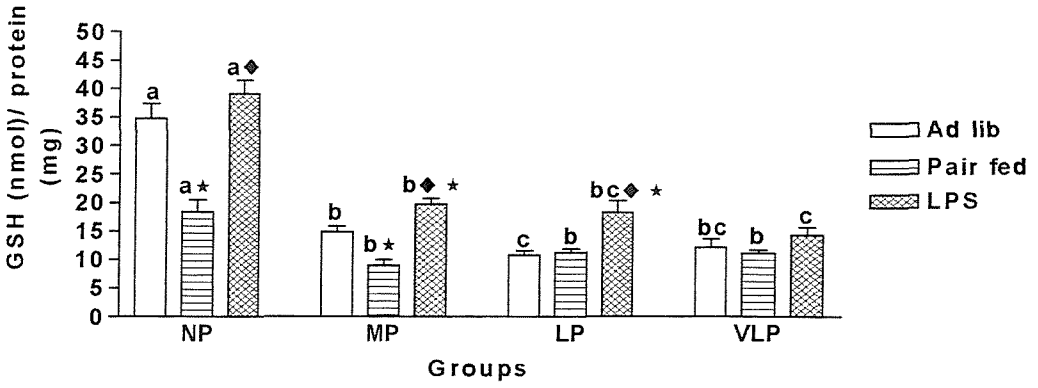
mostly on glutathione to prevent the accumulation of hydrogen peroxide since mitochondria do not contain catalase (Meister, 1991). Looking to the results of the energy efficiency of the various diets in Table 4.3, it can be concluded that the energy utilisation, mostly for building tissues in the case of growing rats, was severely affected as dietary protein content was decreased. Kleiber pointed out that ingestion an imbalance diet, in which a single nutrient was limited, resulted in a decreased efficiency of energy utilisation, and that the metabolisable energy would be lost as heat (Klieber, 1945 & 1961). Forbes *et al.* (1935 & 1944) have shown that as dietary protein content in the diet decreased, the energy efficiency of the diet decreased and heat production increased. For greater heat production more oxygen is utilised. Consequently, H_2O_2 may be produced in greater quantity. This could partly explain the reduction in GSH concentration and the increase in GSH peroxidase activity found in unstressed animals fed the very low-protein diet *ad libitum*. In the pair-fed animals, as the consumption of food is reduced, less oxygen would be consumed by mitochondria, therefore less hydrogen peroxide produced. This may explain the reduction in the activity of hepatic GSH peroxidase in animals with restricted food intakes.

Urea-N excretion was greatly affected by LPS treatment (Table 4.15). Animals exposed to LPS significantly increased the amount of urea-N excretion when compared to values from pair-fed controls. The data indicates that the amino acids released from protein breakdown in peripheral tissue (e.g. muscle), after LPS treatment, was not completely reutilised by the body (e.g. for glutathione and acute phase protein synthesis). The ratio of urea-N excretion to N intake was more severely affected by the reduction in dietary protein concentration than the ratio of ammonia-N to N intake (Table 4.17 & Table 4.18). This is because ammonia excretion was affected primarily by the state of acid base balance (Schepartz, 1973).

The results of the ratio of hepatic GSH to hepatic protein showed that major changes in the proportion of these two important end products of amino acid metabolism were bought about by alterations in protein intake and an inflammatory response. In all treatment groups, animals fed the NP diet had a higher ratio than

animals fed the MP, LP, and VLP diets (Table 4.16 & Figure 4-7). The ratios in the NP and MP groups were more severely affected after food restriction than in the groups fed the low-protein and very low-protein diets. The changes in the ratio indicate that GSH is more sensitive to dietary amino acid supply than liver protein. When an inflammatory stress was applied, the ratio of GSH to protein in liver increased significantly compared with the pair-fed controls given the NP, MP, and LP diets. The ratio increased to a value that is 113%, 120%, 62%, and 28% higher than the corresponding pair-fed controls in the NP, MP, LP, and VLP groups, respectively. The data indicates that after LPS treatment, hepatic GSH responds to a greater extent to amino acid released from peripheral tissues than does protein, however, the magnitude of the increase in the ratio (compared with pair-fed controls) was compromised when dietary protein content was decreased.

Figure 4-7 The ratio of glutathione to protein in liver



Results are mean ± SE, n=6 per treatment group. Means within each treatment group having different letter superscripts differ significantly. ★ indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ◆ indicates a significant difference between pair-fed and LPS-treated groups.

The changes in hepatic GSH content bought about by dietary protein intake and inflammation might have impacted upon the immune system of the animals. In unstressed animals (the *ad libitum* and pair-fed groups) the percentage of lung-neutrophilic cells was affected by dietary protein content. Many studies show that malnutrition compromises several aspects of immunity, such as macrophage function, cell-mediated immune responses, secretory immunoglobulin A antibody

production, and impair cytokine production (Chandra & Kumari, 1994; Doherty, 1994). Furthermore, Gmunder *et al.* (1990), Wilmore *et al.* (1993) and Robinson *et al.* (1993) have shown that glutathione and cysteine are important compounds needed for optimising T-cell function, and enhance lymphocyte proliferation. Thus, feeding a diet restricted in protein and sulphur amino acid content could explain the reduction in the number of neutrophils in lung in animals fed *ad libitum* and in pair-fed controls fed the low-protein dietary groups.

However, when the pro-inflammatory cytokines are activated by LPS treatment, neutrophils in lung increased (not statistically significant) in the low-protein groups compared with the NP and MP groups. The percentage of neutrophils in lung in the LPS-treated animals given the low-protein dietary groups was significantly higher than the corresponding *ad libitum* and pair-fed controls. At the same time the ability of lung to increase GSH concentration in LPS-treated animals was impaired by the low-protein diets, and in the VLP group failed to rise in liver. In the VLP group, GSH concentration in lung, spleen, and thymus of LPS-treated animals was lower than the corresponding pair-fed controls. GSH is a key component of antioxidant defence and an important suppresser of NF-κB activation during inflammation, thereby restraining the production of cytokines, including IL-8 (the neutrophil chemoattractent agent). A failure to increase GSH during inflammation could thus explain the increase in the percentage of neutrophils in lung of LPS-treated animals in the low-protein groups. Pena *et al.* (1999), in an in vivo study, have shown inverse relationship between GSH concentration and monocyte production of IL-8, IL-1, and TNF in whole blood of cirrhotic patients.

The products of the inflammatory response (GSH, albumin, α-1 acid glycoprotein, and liver protein) measured in the present study were not affected to a same degree, when the inflammatory stress of LPS was applied, by dietary protein deficiency. While feeding a protein deficient diet affected hepatic glutathione to a greater extent than other products of inflammation, albumin was affected to a much less extent (Table 4.22).

Table 4.22 A league table of the effects of a protein deficient diet on the products of the inflammatory response

1	**Hepatic glutathione**
2	**Liver protein**
3	**α-1 acid glycoprotein**
4	**Albumin**

The results of the partitioning of sulphur amino acids between their major products (the ratios of GSH to protein in liver, hepatic GSH to SO_4, SO_4 to urea-N, and SO_4 to NH_3-N), show that dietary protein intake exerted a strong influence on the above ratios, in the absence of an inflammatory response and restricted food intake. LPS treatment and restricting food intake by pair feeding did not have a pronounced effect on the ratios, except on the ratio of GSH to protein in liver.

To conclude, the reduction in dietary protein content, using casein as a source of protein, resulted in increasing degree of deficiency of various amino acids, in which sulphur amino acids were the most limiting for growth in three of the diets of low-protein content. The inability of the low protein intakes to support growth and glutathione synthesis may have an adversely synergistic effect. This effect is illustrated by the changes that occurred in growth efficiency, tissue GSH and hepatic GSH peroxidase in the presence and absence of an inflammatory response. In the absence of inflammatory agent the need of the animal to oxidise unwanted nutrients (due to the imbalanced nature of the very low-protein diet) may have imposed an inflammatory stress. In the presence of inflammatory stress, lack of substrate for glutathione synthesis impaired the ability of the animals to maintain antioxidant defences and control the inflammatory process as adequately as in animals fed the diet of highest protein content.

CHAPTER FIVE

5. The effect of graded levels of dietary casein supplemented with methionine to equalise dietary sulphur amino acid content on growth and inflammatory responsiveness in rats

5.1 Introduction

Although graded casein intakes in the previous study resulted in increasing degrees of deficiencies in a number of amino acids (sulphur amino acids, tryptophan...etc.), sulphur amino acids were the most limiting for growth in diets of low protein content (see Table 4.2, chapter 4). Associated with the inadequate sulphur amino acid intake, hepatic glutathione concentration in unstressed animals fed *ad libitum* and in animals given an inflammatory challenge was decreased. The synthesis of glutathione in liver appeared to have been affected to a greater degree than that of protein.

As the precise amino acid requirements for the acute inflammatory response are unknown, the design of the present study was to correct the deficiency of sulphur amino acids for growth and re-examines the effect of deficiencies in the remaining essential amino acids on the acute inflammatory response. The results from the present study would therefore indicate the relative importance of sulphur amino acids and other deficient amino acids in growth and inflammatory responsiveness.

For this purpose, the effect of sulphur amino acid sufficiency, in the presence of deficiency in other essential amino acids, on growth, organ weights, hepatic and lung protein, urinary end products of amino acid metabolism, and glutathione concentrations in liver, lung, spleen and thymus in *ad libitum*, endotoxin-treated animals, and in animals pair-fed the amount of food eaten by the animals receiving the endotoxin were examined. The partitioning of sulphur amino acids into their main end products was also examined indirectly (e.g. as in the previous study) by

examining the ratios of these products (urinary inorganic sulphate and nitrogenous excretion products, and protein and hepatic glutathione). Finally, the number of neutrophils present in lung was counted.

Previous experiments carried out in our laboratory, showed the ability of animals fed low-protein diets supplemented with either methionine or cysteine to enhance glutathione concentration in liver and lung in young growing rats given endotoxin (Hunter & Grimble, 1994). The amount of methionine and cysteine supplemented in Hunter's study was more than three time the amount recommended to support growth in growing rats recommended by the National Research Council (1978) and by Stockland *et al.* (1973) to support growth for young rats, and may have been marginally toxic (Harper *et al.* 1970).

In the present experiment, a physiological amount of the sulphur amino acid, L-methionine, was added to all low-protein diets to be equivalent to the amount of sulphur amino acids present in a usually customary diet (180 g protein + 3 g L-methionine/kg diet). L-methionine but not L-cysteine was chosen for two reasons:

1) Methionine is considered to be essential for growth while cysteine is semi-essential in nature.

2) To minimise the auto-oxidation of cysteine to cystine in the plasma (Estrela *et al.* 1983).

5.2 Sulphur amino acid content of the various diets

All diets were prepared by mixing dry ingredients in a mixer, this was followed by addition of maize oil. To these diets, water was added to make the diets into small biscuits that were dried in oven at 80°C for up to 48-72 hours.

In order to achieve the aim of this study and highlight the importance of sulphur amino acid sufficiency, rats were divided into three different groups all of which received diet containing 65 mmol sulphur amino acids/kg diet as follows:

1) The first group received 180 g protein supplemented with 3 g L-methionine/kg diet. This diet was used as it is a customary diet for young growing rats. This diet

contains 65 mmol sulphur amino acids/kg diet, and this group is called the normal protein + L-methionine (NP+M) group.

2) The second group received 80 g protein supplemented with 6.70 g L-methionine/kg diet. This diet contains 65 mmol sulphur amino acids/kg diet, and this group is called the low-protein diet + L-methionine (LP+M) group.

3) The third group received 60 g protein supplemented with 7.45 g L-methionine/kg diet. This diet contains 65 mmol sulphur amino acids/kg diet, and this group is called the very low-protein diet + L-methionine (VLP+M) group.

- The amount of sulphur amino acids present in the low-protein diets supplemented with methionine (the LP+M and VLP+M diets) used in this study was equivalent to the amount of sulphur amino acids present in the NP+M group. Thus, any influence of sulphur amino acid adequacy in the presence of varying quantities of other amino acids would be apparent by comparison with results from the previous study (see Table 5.1 for diets composition).

Table 5.1 Diets composition (g/kg)

Component	180 g protein/kg	80 g protein /kg	60 g protein/kg
Casein	204	91	68
L-methionine	3	6.70	7.45
Cellulose	100	100	100
Sucrose	319	374	385
Maize starch	319	374	385
Maize oil	30	30	30
Vitamins mix	5	5	5
Minerals mix	20	20	20

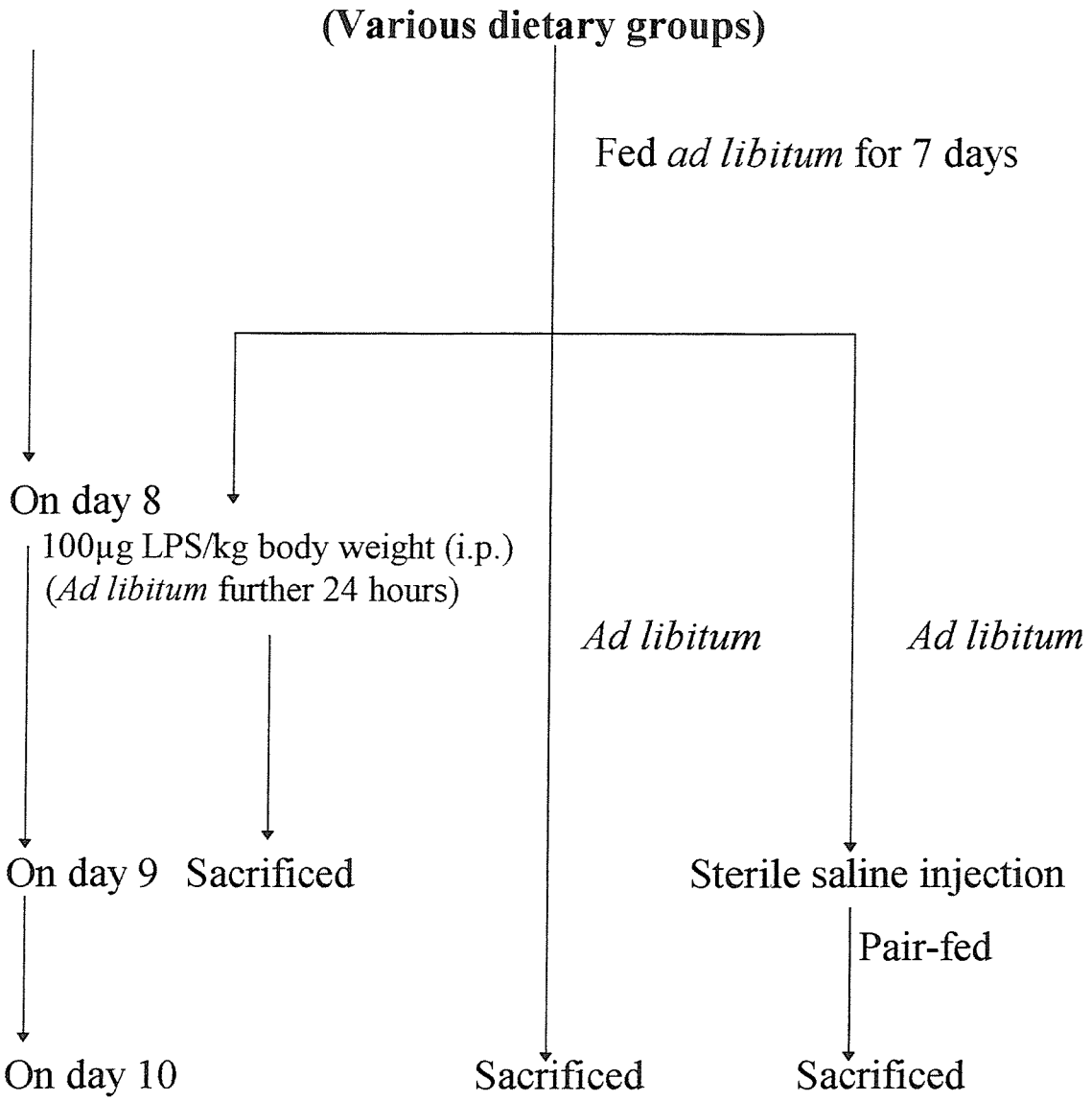
5.3 Experimental protocol

Male weanling wistar rats from Southampton University Medical School Colony weighing 50-70 g were housed individually in plastic metabolic cages and

were maintained at 22 ± 1°C with a 12-12 hour light-dark cycle (Lights on at 7:30 AM).

Rats were divided into three different dietary groups (n=12 for the NP+M group, and n=18 for the LP+M and the VLP+M groups) and fed *ad libitum* the semi-synthetic diets for 7 days. On day 8, animals from each group were subdivided into three treatment groups, and fed, injected and biopsied as described in chapter 4, section 4.3 (see Figure 5-1).

Figure 5-1 Schematic representation of the experimental protocol



5.4 Results

5.4.1 Growth, food intake, sulphur amino acid intake, and organ weights

Average daily weight gain, food intake, sulphur amino acid intake, and food, energy and protein efficiency before starting treatments

There was a progressive reduction in the average daily weight gain as dietary protein content was decreased (Table 5.2). The average daily weight gain of rats fed the LP+M and the VLP+M diets was higher than animals fed the LP and the VLP diets, but without methionine supplementation in the previous study (Table 4.3, Chapter 4). This effect indicates the beneficial effect of introducing methionine in adequate amount on the growth process even though the diet is deficient in protein. However, methionine alone was not enough, even if supplemented in adequate amount to the low-protein diets, to allow rats to reach the average daily weight gain of animals fed the 180 g protein/kg diet supplemented with methionine (as in the present study) or without methionine (as in the previous study). This clearly indicates that there are other limiting amino acids present in the LP+M and VLP+M groups. There was no significant difference in the average daily weight gain between the NP+M group in the present study and the NP group without methionine supplementation in the previous study. It could therefore be concluded that the NP diet was not limiting for growth, even though it is customary to supplement such a diet with sulphur amino acids.

The average daily food intake and (sulphur amino acid intake) was similar between the groups before starting treatments. However, the food efficiency was decreased as protein content in the diet was decreased (Table 5.2). The food efficiency (g weight gain/g of food eaten) of rats fed the LP+M and the VLP+M diets was 0.37 and 0.27 respectively, while in the LP and the VLP groups, without methionine supplementation in the previous study, it was 0.31 and 0.19 respectively. This increase in food efficiency was statistically significant. In the NP+M group, the

food efficiency was 0.44, while in the NP group, in the previous experiment, the food efficiency was 0.49. This decrease in food efficiency was statistically significant.

The values for energy efficiency of various groups showed that this efficiency reduced significantly as dietary protein content in the diet was decreased (Table 5.2). The average daily weight gain for each kJ energy supplied by the diet in the LP+M and VLP+M groups was 20.6 mg and 15 mg of weight gain/kJ eaten respectively, while in the LP and VLP groups, without methionine supplementation in previous study, it was 17 mg and 10.5 mg of weight gain/kJ eaten respectively. This indicates that the addition of methionine to the low-protein diets enhances the energy efficiency. However, in the NP+M group, the energy efficiency was 24.5 mg of weight gain/kJ eaten, while it was 27.3 mg of weight gain/kJ eaten in the NP group without methionine supplementation in the previous study. This decrease in energy efficiency was statistically significant.

The efficiency with which dietary protein was used for growth was higher in animals fed the LP+M and the VLP+M diets than in animals fed the NP+M group (Table 5.2). The protein efficiency (g weight gain/g protein eaten) of rats fed the LP+M and the VLP+M diets was 4.6 and 4.5 respectively, while in the LP and the VLP groups, without methionine supplementation in previous study, it was 3.8 and 3.1 respectively. This increase in protein efficiency was statistically significant. This indicates that addition of methionine to the low-protein diets enhances the utilisation of protein for building tissues, particularly when the diet was severely deficient in protein. However, in the NP+M group, the protein efficiency was 2.5, while in the NP group without methionine supplementation in the previous experiment, the protein efficiency was 2.7. This decreased in the protein efficiency was statistically significant. The slight decrease in the food, energy and protein efficiencies which occurred in the NP+M group in the present study compared with the NP group without methionine in the previous study, was due to the increase in food (protein) intake. the average daily weight gain was similar between the two groups.

Table 5.2 Average daily weight gain, food intake, and food, protein and energy efficiency of rats fed diets of graded casein content supplemented with methionine to equalise dietary sulphur amino acid content, before starting treatments

Dietary groups g protein/kg diet	NP+M 180+M	LP+M 80+M	VLP+M 60+M	one-way ANOVA p-value	 F ratio
weight gain (g)	6.2 ± 0.29^{a}	4.9 ± 0.14^{b}	3.6 ± 0.15^{c}	p=0.000	F=45.9
Food intake (g)	14.0 ± 0.36^{a}	13.4 ± 0.32^{a}	13.4 ± 0.28^{a}	p=0.382	F=0.98
Sulphur amino acids intake (µmol)	910 ± 23^{a}	871 ± 21^{a}	872 ± 18^{a}	p=0.382	F=0.98
Weight gain/ Food intake (g/g)	0.44 ± 0.01^{a}	0.37 ± 0.01^{b}	0.27 ± 0.01^{c}	p=0.000	F=66.7
weight gain/energy intake (mg/kJ)	24.5 ± 0.75^{a}	20.6 ± 0.49^{b}	15.0 ± 0.50^{c}	p=0.000	F=66.7
Weight gain/ protein intake (g/g)	2.5 ± 0.06^{a}	4.6 ± 0.11^{b}	4.5 ± 0.16^{b}	p=0.000	F=68

* Results are presented as mean ± standard error of the mean, n=12 for the NP+M group, and n=18 for the LP+M and the VLP+M groups. Means within each row having different letter superscripts following the number differ significantly.

The effect of LPS on food intake and sulphur amino acid intake

The food intake of animals fed *ad libitum* was similar between the groups. After LPS treatment, a reduction in food (and sulphur amino acid intake) occurred in all the groups compared with animals fed *ad libitum*. This reduction achieved statistical significant in rats fed the LP+M and the VLP+M diets (Table 5.3).

Table 5.3 The effect of LPS on food intake and sulphur amino acid intake (S.A.A.I.) of rats fed diets of graded casein content supplemented with methionine to equalise dietary sulphur amino acid content

Dietary groups g protein/kg diet	NP+M 180+M	LP+M 80+M	VLP+M 60+M	two-way ANOVA p-value F ratio
Food intake				
ad libitum	15.7 ± 1.48[a]	13.9 ± 1.44[a]	15.9 ± 1.06[a]	D=0.021 F=4.27
pair-fed	12.0 ± 1.05[a]	7.7 ± 1.29[b]*	7.9 ± 1.29[b]*	T=0.000 F=16.37
LPS	12.4 ± 1.40[a]	8.1 ± 1.75[a]*	8.4 ± 1.35[a]*	I=0.585 F=0.72
S.A.A.I.				
ad libitum	1020 ± 96[a]	908 ± 94[a]	1035 ± 69[a]	D=0.021 F=4.27
pair-fed	784 ± 68.3[a]	504 ± 84[b]*	514 ± 84[b]*	T=0.000 F=16.37
LPS	807 ± 91[a]	528 ± 114[a]*	545 ± 88[a]*	I=0.585 F=0.72

-Results are presented as mean ± standard error of the mean, n=4 per treatment group for animals fed the NP+M diet, and n=6 per treatment group for animals fed the LP+M and the VLP+M diets. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

Final body weight

The results of the final body weight of animals showed that in all dietary groups there was a reduction in the final body weight as dietary protein content was decreased (Table 5.4). This reduction in body weight was statistically significant when comparing the LP+M and the VLP+M groups with the NP+M groups in the animals fed *ad libitum* and in pair-fed controls.

In the LP+M and the VLP+M groups, the final body weight of the LPS-treated animals was significantly lower than the corresponding animals fed *ad libitum*.

Table 5.4 Final body weight of rats fed diets of graded casein content supplemented with methionine to equalise dietary sulphur amino acid content

Dietary groups g protein/kg diet	NP+M 180+M	LP+M 80+M	VLP+M 60+M	two-way ANOVA p-value F ratio
Final body weight (g)				
ad libitum	115.0 ± 4.7 [a]	100.0 ± 3.6[b]	91.8 ± 3.1[b]	D=0.000 F=29.32
pair-fed	104.4 ± 3.6 [a]	90.1 ± 3.0[b]	83.3 ± 2.2[b]	T=0.000 F=12.29
LPS	102.6 ± 8.0 [a]	84.2 ± 2.7 [a]*	76.4 ± 2.1 [a]*	I=0.970 F=0.13

-Results are presented as mean ± standard error of the mean, n=4 per treatment group for animals fed the NP+M diet, and n=6 per treatment group for animals fed the LP+M and the VLP+M diets. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

Organ weights

a) Total liver weight

The results for the total liver weight can be seen in Table 5.5. Due to diet, no significant differences in the total liver weights were found between the groups.

Due to treatments, in all the groups, the total liver weight of the pair-fed controls was significantly lower than in the *ad libitum*-fed animals. The total liver weight of animals fed the VLP+M diet and given LPS was significantly higher than the corresponding pair-fed controls, and lower than the corresponding animals fed *ad libitum*.

b) Relative liver weight

Table 5.5 shows that in animals fed *ad libitum*, there was an increase in the relative liver weight (g/kg body weight) when dietary protein content was decreased to 60 g/kg diet. The increase was statistically significant when comparing the VLP+M group with the NP+M group. The mean values for the relative liver weight in the animals fed *ad libitum* was higher than in the pair-fed controls in all dietary groups, but the difference was statistically significant in the NP+M and VLP+M

groups only. In the pair-fed animals, the mean values was higher in the LP+M and VLP+M groups compared with the NP+M group, but this difference was not statistically significant. In the LPS-treated animals, the relative liver weight was significantly higher in the VLP+M groups compared with the NP+M group.

Due to treatments, in all dietary groups, the LPS-treated animals had a higher relative liver weight compared with the corresponding pair-fed controls.

c) Total lung weight

In animals fed *ad libitum*, pair-fed controls and LPS-treated animals, the total lung weight was significantly lower in the VLP+M group compared to the NP+M group.

No significant difference in the total lung weight was found between the groups due to treatments (Table 5.5).

d) Relative lung weight

Neither diet nor treatments caused a significant alteration in the relative lung weight (g/kg body weight) (Table 5.5).

Table 5.5 Organ weights of rats fed diets of graded casein content supplemented with methionine to equalise dietary sulphur amino acid content, after starting treatments

Dietary groups g protein/kg diet	NP+M 180+M	LP+M 80+M	VLP+M 60+M	two-way ANOVA p-value F ratio
Liver weight (g)				
ad libitum	6.12 ± 0.56 ^{a}	5.72 ± 0.40 ^{a}	5.94 ± 0.44 ^{a}	D=0.383 F=0.98
pair-fed	4.25 ± 0.16 ^{a} *	4.13 ± 0.33 ^{a} *	3.93 ± 0.23 ^{a} *	T=0.000 F=20.06
LPS	5.33 ± 0.40 ^{a}	4.87 ± 0.31 ^{a}	4.62 ± 0.14 ^{a} ♠*	I=0.914 F=0.24
Relative liver weight (g/kg body weight)				
ad libitum	53.0 ± 2.8 ^{a}	55.0 ± 4.1 $^{a\,b}$	64.3 ± 2.8 ^{b}	D=0.003 F=6.97
pair-fed	40.9 ± 2.4 ^{a}*	45.6 ± 2.7 ^{a}	47.1 ± 2.5 ^{a}*	T=0.000 F=20.24
LPS	52.0 ± 0.6 ^{a}♠	57.7 ± 2.6 $^{a\,b}$♠	60.5 ± 1.4 ^{b}♠	I=0.608 F=0.68
Lung weight (mg)				
ad libitum	867 ± 18 ^{a}	857 ± 85 $^{a\,b}$	742 ± 32 ^{b}	D=0.003 F=6.96
pair-fed	830 ± 29 ^{a}	787 ± 43 $^{a\,b}$	733 ± 20 ^{b}	T=0.126 F=2.18
LPS	828 ± 21 ^{a}	753 ± 26 ^{a}	669 ± 13 ^{b}	I=0.889 F=0.28
Relative lung weight (g/kg body weight)				
ad libitum	7.56 ± 0.21 ^{a}	8.57 ± 0.85 ^{a}	8.09 ± 0.24 ^{a}	D=0.061 F=3.00
pair-fed	7.97 ± 0.35 ^{a}	8.71 ± 0.24 ^{a}	8.83 ± 0.28 ^{a}	T=0.237 F=1.49
LPS	8.18 ± 0.46 ^{a}	8.95 ± 0.20 ^{a}	8.78 ± 0.22 ^{a}	I=0.960 F=0.15
Spleen weight (mg)				
ad libitum	565 ± 21 ^{a}	413 ± 23 ^{b}	308 ± 16 ^{c}	D=0.000 F=63.06
pair-fed	563 ± 54 ^{a}	368 ± 29 ^{b}	269 ± 15 ^{c}	T=0.347 F=1.09
LPS	497 ± 39 ^{a}	393 ± 16 ^{a}	322 ± 16 ^{b}	I=0.254 F=1.39
Relative spleen weight (g/kg body weight)				
ad libitum	4.95 ± 0.30 ^{a}	4.12 ± 0.16 ^{a}	3.35 ± 0.10 ^{b}	D=0.000 F=40.36
pair-fed	5.36 ± 0.34 ^{a}	4.06 ± 0.23 ^{b}	3.23 ± 0.13 ^{c}	I=0.053 F=3.16
LPS	4.85 ± 0.14 ^{a}	4.68 ± 0.19 ^{a}	4.05 ± 0.17 ^{b}♠*	I=0.038 F=2.83
Thymus weight (mg)				
ad libitum	749 ± 65 ^{a}	578 ± 38 $^{a\,b}$	533 ± 13 ^{b}	D=0.000 F=14.89
pair-fed	645 ± 77 ^{a}	512 ± 39 ^{a}	456 ± 53 ^{a}	T=0.009 F=5.26
LPS	615 ± 59 ^{a}	478 ± 20 ^{a}	439 ± 20 ^{a}*	I=0.989 F=0.08
Relative thymus weight (g/kg body weight)				
ad libitum	6.49 ± 0.38 ^{a}	5.76 ± 0.24 ^{a}	5.84 ± 0.24 ^{a}	D=0.259 F=1.40
pair-fed	6.14 ± 0.59 ^{a}	6.65 ± 0.30 ^{a}	5.46 ± 0.64 ^{a}	T=0.681 F=0.39
LPS	5.98 ± 0.16 ^{a}	5.70 ± 0.27 ^{a}	5.80 ± 0.41 ^{a}	I=0.959 F=0.16

-Results are presented as mean ± standard error of the mean, n=4 per treatment group for animals fed the NP+M diet, and n=6 per treatment group for animals fed the LP+M and the VLP+M diets. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

e) Total spleen weight

In animals fed *ad libitum*, and in pair-fed controls, the total spleen weight significantly reduced as dietary protein content was decreased. In the LPS-treated animals, there was a reduction in the total spleen weight as dietary protein content was decreased. This was statistically significant difference between the NP+M group and the VLP+M group.

No significant difference in the total spleen weight was found between the groups due to treatments (Table 5.5).

f) Relative spleen weight

From Table 5.5 it can be seen that in the *ad libitum* fed and in LPS-treated animals, the VLP+M group had significantly lower relative spleen weight (g/kg body weight) compared with the NP+M group. In the pair-fed animals, the relative spleen weight decreased significantly as dietary content was decreased.

Due to the interaction between diet and treatments, the LPS-treated animals fed the VLP+M diet had a higher relative spleen weight compared with the corresponding *ad libitum* and pair-fed controls.

Addition of methionine to the 80 g protein and 60 g protein/kg diet had no effect upon relative spleen weight, unlike its influence on the relative liver weight.

g) Total thymus weight

In animals fed *ad libitum*, LPS-treated rats and pair-fed controls, there was a trend for a reduction in the total thymus weight as dietary protein content was decreased. This was statistically significant in the *ad libitum*-fed animals when comparing the NP+M group with the VLP+M group (Table 5.5).

h) Relative thymus weight

Neither diet nor treatments caused an alteration in the relative thymus weight (g/kg body weight) between the groups (Table 5.5).

Addition of methionine to the diets had no effect on the relative thymus weight compared with animals fed the same diets but without methionine supplementation in the previous chapter.

5.4.2 Glutathione concentration, and glutathione peroxidase and reductase activities

Glutathione concentration

a) Liver glutathione concentration

Hepatic glutathione (GSH) concentration was influenced by dietary protein content supplemented with methionine, LPS treatment and food intake (Table 5.6).

In animals fed *ad libitum*, hepatic GSH concentration of the VLP+M group was significantly higher than the NP+M and LP+M groups despite a similar sulphur amino acid intake. Pair feeding reduced hepatic GSH content but, as in the *ad libitum*-fed animals, hepatic GSH concentration of the VLP+M group was significantly higher than in the NP+M and LP+M groups. After LPS treatment, no difference in GSH concentration was found between the groups.

Comparing the LPS-treated animals with the pair-fed controls, Table 5.6 shows that in all dietary groups, hepatic GSH concentration was increased dramatically in the LPS-treated animals compared with the corresponding pair-fed controls.

Addition of methionine to the NP diet slightly increased GSH concentrations in the *ad libitum* and LPS-treated animals, but this increase was not statistically significant. Addition of methionine to the low-protein diets increased GSH concentrations in all treatment groups compared with animals fed the same diets but without methionine supplementation in the previous chapter (Table 4.7, chapter 4). However, this increase in hepatic GSH concentration, after addition of methionine to the low-protein diets, was greater in the *ad libitum* and in LPS-treated animals than in the pair-fed controls.

b) Lung glutathione concentration

No difference in lung GSH concentration was found due to the effect of diet. However, LPS treatment and food restriction influenced lung GSH concentration (Table 5.6).

Restriction in food intake caused a significant reduction in lung GSH concentration only in the NP+M group. Table 5.6 shows that in all dietary groups, a significant increase in lung GSH concentration was found in the LPS-treated animals compared with the pair-fed controls. This contrasted with the effect of LPS on lung GSH in animals receiving the LP and VLP diets where no increase and a fall in GSH concentration occurred respectively (Table 4.7, chapter 4). In the NP+M and the VLP+M groups, lung GSH concentration in the LPS-treated animals was also significantly higher than in the *ad libitum*-fed animals.

c) Spleen glutathione concentration

Although animals fed the VLP+M diet were able to maintain hepatic and lung GSH concentration to the same levels of other dietary groups, they were unable to maintain spleen GSH concentration (Table 5.6).

In the *ad libitum* and LPS-treated animals, spleen GSH concentration was significantly lower in the VLP+M group compared to the NP+M group.

Neither LPS treatment, nor restriction in food intake caused a significant alteration in spleen GSH concentration, this contrasted to the effect of LPS on animals consuming the VLP diet, without methionine supplementation. In these animals, GSH fell after LPS treatment (Table 4.7, chapter 4).

d) Thymus glutathione concentration

Table 5.6 shows that in animals fed *ad libitum*, and in LPS-treated animals, GSH concentration in thymus was lower in the VLP+M group compared with the NP+M group. After pair feeding, no difference in thymus GSH concentration was found between the groups.

Neither LPS treatment nor restriction in food intake caused a significant alteration in thymus GSH concentration.

Again supplementing the VLP diet with methionine prevented the fall in GSH when the LPS stimulus was applied to the rats.

Table 5.6 Glutathione concentration in liver, lung, spleen and thymus of rats fed diets of graded casein content supplemented with methionine to equalise dietary sulphur amino acid content, after starting treatments (µmol/g tissue)

Dietary groups g protein/kg diet	NP+M 180+M	LP+M 80+M	VLP+M 60+M	two-way ANOVA p-value	 F ratio
Liver					
ad libitum	6.18 ± 0.19^{a}	5.66 ± 0.39^{a}	7.86 ± 0.31^{b}	D=0.000	F=15.07
pair-fed	2.63 ± 0.39^{a}*	3.06 ± 0.38^{a}*	4.65 ± 0.55^{b}*	T=0.000	F=92.25
LPS	6.84 ± 0.15^{a} *♠	7.45 ± 0.22^{a}*♠	7.52 ± 0.26^{a} ♠	I=0.034	F=2.89
Lung					
ad libitum	1.90 ± 0.07^{a}	1.81 ± 0.04^{a}	1.78 ± 0.10^{a}	D=0.506	F=0.69
pair-fed	1.57 ± 0.02^{a} *	1.70 ± 0.06^{a}	1.71 ± 0.06^{a}	T=0.000	F=23.66
LPS	2.20 ± 0.11^{a} ♠	2.05 ± 0.09^{a} ♠	1.96 ± 0.05^{a} ♠	I= 0.181	F=1.65
Spleen					
ad libitum	3.26 ± 0.02^{a}	2.87 ± 0.25^{ab}	2.64 ± 0.15^{b}	D=0.004	F=6.55
pair-fed	3.00 ± 0.09^{a}	2.99 ± 0.11^{a}	2.68 ± 0.10^{a}	T= 0.190	F=1.73
LPS	3.31 ± 0.11^{a}	3.13 ± 0.16^{ab}	2.88 ± 0.06^{b}	I=0.795	F=0.42
Thymus					
ad libitum	2.12 ± 0.03^{a}	1.90 ± 0.13^{ab}	1.89 ± 0.08^{b}	D=0.019	F=4.42
pair-fed	2.03 ± 0.05^{a}	2.02 ± 0.09^{a}	1.92 ± 0.11^{a}	T=0.140	F=2.07
LPS	2.35 ± 0.07^{a}	2.04 ± 0.13^{ab}	1.97 ± 0.03^{b}	I=0.616	F=0.67

-Results are presented as mean ± standard error of the mean, n=4 per treatment group for animals fed the NP+M diet, and n=6 per treatment group for animals fed the LP+M and the VLP+M diets. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

Glutathione peroxidase activity

a) Glutathione peroxidase activity in liver

The results of the liver glutathione peroxidase (GPx) activity can be seen in Table 5.7. Due to diet, hepatic GPx activity in the VLP+M group was significantly higher than in the NP+M group, in both animals fed *ad libitum* and after LPS

treatment, as in the previous study. However, addition of methionine to the NP, LP and VLP diets in the *ad libitum* and in LPS-treated animals, resulted in a fall in liver GPx activity compared with the previous experiment (Table 4.8, chapter 4). In the pair-fed animals, no difference in GPx activity was found between the groups.

Due to treatments, in the VLP+M group, GPx activity of the LPS treated and *ad libitum*-fed animals was significantly higher than the corresponding pair-fed controls, as in the previous study.

b) Glutathione peroxidase activity in lung, spleen, and thymus

No difference in GPx activity was found in the above organs due to the effect of diet, LPS treatment, or restriction in food intake (Table 5.7). The lack of effect also occurred in the previous study (Table 4.8, chapter 4).

Table 5.7 Glutathione peroxidase activity in liver, lung, spleen, and thymus of rats fed diets of graded casein content supplemented with methionine to equalise dietary sulphur amino acid content, after starting treatments (units/g protein)

Dietary groups g protein/kg diet	NP+M 180+M	LP+M 80+M	VLP+M 60+M	two-way ANOVA p-value	 F ratio
liver					
ad libitum	28.8 ± 1.5 [a]	33.4 ± 2.2 [ab]	39.7 ± 2.5 [b]	D=0.009	F=5.61
pair-fed	28.7 ± 1.8 [a]	29.7 ± 2.2 [a]	27.0 ± 2.8 [a]*	T=0.006	F=6.27
LPS	30.5 ± 1.0 [a]	32.1 ± 2.0 [ab]	38.9 ± 2.5 [b]♠	I=0.050	F=2.74
Lung					
ad libitum	60.1 ± 1.8 [a]	60.7 ± 1.7 [a]	64.1 ± 2.2 [a]	D=0.160	F=1.96
pair-fed	61.4 ± 2.3 [a]	58.7 ± 1.7 [a]	60.2 ± 1.1 [a]	T=0.460	F= 0.80
LPS	58.2 ± 1.6 [a]	59.7 ± 0.4 [a]	62.3 ± 1.7 [a]	I=0.511	F=0.84
Spleen					
ad libitum	63.9 ± 1.5 [a]	65.8 ± 1.1 [a]	66.9 ± 0.8 [a]	D=0.089	F=2.65
pair-fed	67.3 ± 2.0 [a]	60.6 ± 1.4 [a]	62.1 ± 1.3 [a]	T=0.201	F=1.70
LPS	64.8 ± 2.2 [a]	61.4 ± 0.7 [a]	65.2 ± 1.5 [a]	I=0.052	F=2.70
Thymus					
ad libitum	60.1 ± 1.2 [a]	61.4 ± 3.3 [a]	64.4 ± 2.0 [a]	D=0.375	F=1.02
pair-fed	62.3 ± 2.2 [a]	63.5 ± 4.3 [a]	63.2 ± 2.2 [a]	T=0.847	F=0.17
LPS	61.3 ± 1.4 [a]	61.1 ± 0.9 [a]	64.0 ± 1.5 [a]	I=0.934	F=0.20

-Results are presented as mean ± standard error of the mean, n=4 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

Glutathione reductase activity

a) Glutathione reductase activity in liver

Glutathione reductase (GR) activity in liver was influenced by dietary protein intake supplemented with methionine in animals fed *ad libitum* and in LPS-treated animals, but not in pair-fed controls (Table 5.8).

In the *ad libitum* fed and in LPS-treated animals, GR activity in liver was significantly higher in the VLP+M group compared with the NP+M and LP+M groups. In the pair-fed controls, no significant difference in GR activity was found between the groups.

In the VLP+M group, there was a trend for an increase in GR activity in the LPS-treated rats and in the *ad libitum*-fed animals compared to the corresponding pair-fed controls, but this difference was not statistically significant.

Addition of methionine to the diets had no effect on the activity of GR compared with the previous experiment (Table 4.9, chapter 4).

Glutathione reductase activity in lung, spleen, and thymus

As in the previous experiment, Neither the reduction in protein content in the diet nor treatments caused an alteration in the GR activity (Table 5.8).

Table 5.8 Glutathione reductase activity in liver, lung, spleen and thymus of rats fed diets of graded casein content supplemented with methionine to equalise dietary sulphur amino acid content, after starting treatments (units/mg protein)

Dietary groups g protein/kg diet	NP+M 180+M	LP+M 80+M	VLP+M 60+M	two-way ANOVA p-value F ratio
Liver				
ad libitum	20.2 ± 1.4 [a]	19.6 ± 2.4[a]	29.1 ± 1.6[b]	D=0.000 F=12.06
pair-fed	17.4 ± 3.4 [a]	18.7 ± 2.1 [a]	18.2 ± 3.3 [a]	T=0.331 F=1.15
LPS	16.5 ± 0.8[a]	18.1 ± 1.0[a]	28.8 ± 1.4[b]	I=0.330 F=1.21
Lung				
ad libitum	22.6 ± 0.9 [a]	18.6 ± 1.1 [a]	22.1 ± 1.3 [a]	D=0.569 F=0.58
pair-fed	21.4 ± 0.9 [a]	21.6 ± 1.3 [a]	19.3 ± 1.0 [a]	T=0.511 F=0.69
LPS	19.2 ± 1.5 [a]	20.1 ± 1.0 [a]	20.9 ± 0.8 [a]	I=0.053 F=2.67
Spleen				
ad libitum	17.0 ± 2.2 [a]	17.5 ± 0.9 [a]	17.0 ± 1.2 [a]	D=0.069 F=2.97
pair-fed	16.9 ± 0.9 [a]	16.7 ± 0.4 [a]	17.2 ± 1.0 [a]	T=0.875 F=0.13
LPS	13.5 ± 0.9 [a]	19.7 ± 1.2 [a]	19.2 ± 1.5 [a]	I=0.065 F=2.52
Thymus				
ad libitum	17.1 ± 1.1 [a]	18.7 ± 2.1 [a]	23.4 ± 1.3 [a]	D=0.058 F=3.16
pair-fed	19.2 ± 0.8 [a]	18.3 ± 1.7 [a]	19.9 ± 1.7 [a]	T=0.461 F=0.80
LPS	20.9 ± 2.0 [a]	19.5 ± 1.2 [a]	21.6 ± 1.0 [a]	I=0.352 F=1.15

-Results are presented as mean ± standard error of the mean, n=4 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

5.4.3 Protein in liver and lung

a) Liver protein concentration

Hepatic protein concentration was reduced when the protein concentration in the diet was decreased to 80 and 60 g/kg diet in animals fed *ad libitum* (Table 5.9). In the pair-fed animals, no difference was found between the groups. In the LPS-treated animals, the concentration was significantly higher in the NP+M group compared with animals fed the low-protein diets.

Due to treatments, in the NP+M group, protein concentration was significantly higher in the LPS treated and pair-fed animals compared with the corresponding animals fed *ad libitum*. In the VLP+M group, protein concentration was higher in the pair-fed animals compared with the corresponding LPS-treated rats and *ad libitum*-fed animals.

b) Total liver protein

The total liver protein content was influenced by dietary protein intake, food intake and LPS treatment (Table 5.9). In general, the total liver protein, among animals of various dietary and treatment groups, varied due to the difference in the size of livers (Table 5.5).

The result of the total liver protein content showed that in animals fed *ad libitum*, there was a trend for a reduction in the total liver protein as dietary protein content was decreased, but this reduction was not statistically significant. In the pair-fed animals, the total liver protein was lower in the VLP+M group compared to the NP+M group. In the LPS-treated animals, as in the animals fed *ad libitum*, there was a trend for a reduction in the total liver protein as dietary protein content was decreased. This was statistically significant when comparing the NP+M group with the VLP+M group.

Due to treatments, both in the NP+M and in the LP+M groups, there was a trend for an increase in the total liver protein after LPS treatment compared with the pair-fed controls. This increase was statistically significant in the NP+M group. In all dietary groups, the total liver protein in pair-fed animals was lower than in the *ad libitum* fed animals.

c) Total liver protein/kg body weight

The results of the liver protein, as a proportion of body weight, showed that all dietary groups were able to increase the relative liver protein after LPS treatment (Table 5.9).

No significant difference between the groups was found due to differences in diet. In all the groups, the liver protein, as a proportion of body weight, was lower in the pair-fed animals compared with the animals fed *ad libitum.* The relative liver protein increased significantly in the NP+M group after LPS treatment when compared with the corresponding *ad libitum* and pair-fed animals, and it was increased significantly in the LP+M and the VLP+M groups after LPS treatment compared with the corresponding pair-fed animals.

Addition of methionine to the low and very low-protein diets, allowed the relative liver protein to be increased in response to LPS, while in the previous study (Table 4.10, chapter 4), animals fed the same diets, but without added methionine, the relative liver protein did not increase in response to LPS.

Table 5.9 Protein in liver and lung of rats fed diets of graded casein content supplemented with methionine to equalise dietary sulphur amino acid content, after starting treatments

Dietary groups g protein/kg diet	NP+M 180+M	LP+M 80+M	VLP+M 60+M	two-way ANOVA p-value	 F ratio
Liver protein (mg/g liver)					
ad libitum	153.5 ± 1.2 ^{a}	140.6 ± 2.3 ^{b}	126.5 ± 5.7 ^{b}	D=0.000	F=11.56
pair-fed	162.2 ± 2.6 ^{a}*	149.8 ± 5.1 ^{a}	156.8 ± 6.1 ^{a}*	T=0.000	F=9.76
LPS	161.8 ± 2.5 ^{a}*	149.3 ± 3.7 ^{b}	139.9 ± 3.8 ^{b}♠	I=0.081	F=2.25
Total liver protein (mg/total liver)					
ad libitum	897 ± 48 ^{a}	802 ± 50 ^{a}	744 ± 42 ^{a}	D=0.000	F=9.90
pair-fed	696 ± 23 ^{a}*	611 ± 34 ^{ab}*	609 ± 20 ^{b}*	T=0.000	F=14.20
LPS	859 ± 54 ^{a}♠	727 ± 49 ^{ab}	645 ± 20 ^{b}	I=0.639	F=0.64
Relative liver protein (g/kg body weight)					
ad libitum	7.79 ± 0.12	7.99 ± 0.30	8.09 ± 0.26	D=0.370	F=1.02
pair-fed	6.69 ± 0.33*	6.77 ± 0.25*	7.31 ± 0.11*	T=0.000	F=26.29
LPS	8.41 ± 0.18♠*	8.59 ± 0.37♠	8.46 ± 0.22♠	I=0.715	F=0.53
Lung protein (mg/g lung)					
ad libitum	73.1 ± 2.8 ^{a}	69.3 ± 2.5 ^{a}	75.9 ± 2.9 ^{a}	D=0.060	F=3.03
pair-fed	73.5 ± 1.7 ^{a}	70.0 ± 1.6 ^{a}	70.7 ± 2.0 ^{a}	T=0.185	F=1.76
LPS	75.1 ± 2.4 ^{a}	72.4 ± 1.64 ^{a}	77.2 ± 2.1 ^{a}	I=0.640	F=0.64
Total lung protein (mg/total lung)					
ad libitum	63.5 ± 3.3 ^{a}	62.9 ± 3.4 ^{a}	56.1 ± 2.4 ^{a}	D=0.002	F=7.45
pair-fed	61.1 ± 3.0 ^{a}	55.0 ± 3.1 ^{ab}	51.8 ± 1.8 ^{b}	T=0.069	F=2.86
LPS	62.2 ± 3.0 ^{a}	54.7 ± 3.0 ^{ab}	51.6 ± 1.3 ^{b}	I=0.788	F=0.43
Relative lung protein (g/kg body weight)					
ad libitum	0.55 ± 0.01 ^{a}	0.64 ± 0.05 ^{a}	0.61 ± 0.02 ^{a}	D=0.089	F=2.58
pair-fed	0.59 ± 0.03 ^{a}	0.61 ± 0.20 ^{a}	0.62 ± 0.03 ^{a}	T=0.146	F=2.02
LPS	0.61 ± 0.02 ^{a}	0.65 ± 0.02 ^{a}	0.68 ± 0.02 ^{a}	I=0.811	F=0.39

-Results are presented as mean ± standard error of the mean, n=4 per treatment group for animals fed the NP+M diet, and n=6 per treatment group for animals fed the LP+M and the VLP+M diets. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

d) Lung protein concentration

Animals were able to maintain lung protein concentration even under severely protein deficient conditions. No significant difference in lung protein concentration between the groups was found due to differences in diet or treatments (Table 5.9).

e) Total lung protein

In *ad libitum*-fed animals, the mean value was lower in the VLP+M group compared with the NP+M and the LP+M groups. This difference was not statistically significant. In the LPS treated and in pair-fed animals, there was a trend for a reduction in the total lung protein as dietary protein content was decreased. This difference was statistically significant when comparing the NP+M group with the VLP+M group (Table 5.9).

f) Total lung protein/kg body weight

The results of the lung protein, as a proportion of body weight, showed that all animals of various dietary and treatment groups were able to maintain the ratio. No significant difference between the groups was found due to differences in diet or treatments (Table 5.9).

Addition of methionine to the low and very low-protein diets had no effect on the relative lung protein compared with animals receiving the same concentration of protein, but without methionine supplementation in the previous study.

5.4.4 Serum concentrations of α-1 acid glycoprotein and albumin

α-1 acid glycoprotein

In all dietary groups, rats exposed to the inflammatory stimulus of LPS dramatically increased their plasma acute phase protein, α-1 acid glycoprotein (Table 5.10).

No difference in the serum level of α-1 acid glycoprotein was found between the groups due to diet.

In the LPS-treated animals, addition of methionine to the low and very low-protein diets enhanced the serum level of α-1 acid glycoprotein over the level found in animals fed the same diets, but without methionine supplementation in the previous chapter (Table 4.11, chapter 4).

Table 5.10 Serum concentrations of α-1 acid glycoprotein and albumin of rats fed diets of graded casein content supplemented with methionine to equalise dietary sulphur amino acid content, after starting treatments

Groups	NP+M 180+M	LP+M 80+M	VLP+M 60+M	two-way ANOVA p value	F ratio
α-1 acid glycoprotein (μg/ml)					
ad libitum	86 ± 11 [a]	82 ± 5 [a]	82 ± 9 [a]	D=0.768	F=0.27
pair-fed	90 ± 7 [a]	95 ± 8 [a]	88 ± 12 [a]	T=0.000	F=170.7
LPS	835 ± 129 [a]♠*	931 ± 159 [a]♠*	946 ± 6 [a]♠*	I=0.890	F=0.28
Albumin (mg/ml)					
ad libitum	34.2 ± 0.57 [a]	30.2 ± 0.60 [b]	29.5 ± 0.57 [b]	D=0.000	F=23.99
pair-fed	33.9 ± 0.39 [a]	33.4 ± 0.71 [a]*	30.8 ± 0.72 [b]	T=0.004	F=6.51
LPS	33.1 ± 0.71 [a]	29.9 ± 0.66 [b]♠	29.5 ± 0.61 [b]	I=0.125	F=1.93

-Results are presented as mean ± standard error of the mean, n=4 per treatment group for animals fed the NP+M diet, and n=6 per treatment group for animals fed the LP+M and the VLP+M diets. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

Albumin

Feeding a diet low in protein content decreased serum albumin concentration (Table 5.10). In all dietary groups, albumin concentration in the LPS-treated animals slightly decreased compared with the pair-fed controls. This was statistically significant in animals fed the LP+M diet.

Addition of methionine to the NP, LP, and VLP groups had no pronounced effect on the serum level of albumin (see Table 4.11, chapter 4).

5.4.5 Inorganic sulphate excretion, and urinary nitrogenous end products of amino acid metabolism: Urea-N and ammonia-N excretion

Inorganic sulphate excretion, and inorganic sulphate excretion as a percentage of sulphur amino acid intake-average of two days before starting treatments

Inorganic sulphate excretion (SO_4) and SO_4 excretion, as a percentage of sulphur amino acid intake, were increased significantly in the VLP+M group compared with the NP+M group (Table 5.11). SO_4 excretion, as a percentage of sulphur amino acid intake, was reduced by addition of methionine to the low and very low-protein diets compared with data from animals receiving the same amount of casein but without added methionine, in the previous study (Table 4.12, chapter 4).

Table 5.11 Inorganic sulphate excretion, and inorganic sulphate excretion as a percentage of sulphur amino acid intake (S.A.A.I.) of rats fed diets of graded casein content supplemented with methionine to equalise dietary sulphur amino acid content-average of two days before starting treatments (μmol/day)

Dietary groups g protein/kg diet	NP+M 180+M	LP+M 80+M	VLP+M 60+M	one-way ANOVA p-value F ratio
SO_4 excretion	322 ± 13^{a}	352 ± 10^{ab}	385 ± 15^{b}	p=0.008 F=5.62
(SO_4 excretion/ S.A.A.I.) x 100	34.3 ± 1^{a}	37.4 ± 1^{ab}	40.0 ± 1^{b}	p=0.034 F=3.75

* Results are presented as mean ± standard error of the mean, n=12 per group. Means within each row having different letter superscripts following the number differ significantly.

Inorganic sulphate excretion after starting treatments

The results of SO_4 excretion, after starting treatments, can be seen in Table 5.12. In the animals fed *ad libitum*, SO_4 excretion was significantly higher in the VLP+M group compared with the NP+M group. In the LPS-treated and pair-fed animals, no difference in SO_4 excretion was found between the groups.

Due to treatments, SO_4 excretion of the LPS treated and pair-fed animals fed the LP+M and VLP+M diets reduced significantly compared with the corresponding

animals fed *ad libitum*-fed. A significant increase in SO_4 excretion was found in the LPS-treated animals compared with the pair-fed controls given the NP+M group, while in other dietary groups no difference was found.

Table 5.12 Inorganic sulphate excretion of rats fed diets of graded casein content supplemented with methionine to equalise dietary sulphur amino acid content, after starting treatments (μmol/day)

Dietary groups	NP+M	LP+M	VLP+M	two-way ANOVA	
g protein/kg diet	180+M	80+M	60+M	p-value	F ratio
ad libitum	321 ± 17 ^{a}	345 ± 13 ^{ab}	383 ± 10 ^{b}	D= 0.040	F=3.59
pair-fed	254 ± 70 ^{a*}	248 ± 18 ^{a*}	271 ± 10 ^{a*}	T= 0.000	F=37.95
LPS	303 ± 10♠ ^{a}	248 ± 17^{a*}	277 ± 12^{a*}	I=0.028	F=3.21

-Results are presented as mean ± standard error of the mean, n=4 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly.* indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

Ammonia-N and urea-N excretion-average of two days before starting treatments

A significant reduction in ammonia-N excretion was found in the LP+M and the VLP+M groups compared with the NP+M group (Table 5.13). When the protein content in the diet was decreased a significant reduction in urea-N excretion occurred (Table 5.13).

When ammonia-N and urea-N excretion in the present study was compared with the previous study, in which animals fed diets contained same concentrations of protein but without methionine supplementation, it can be seen that the addition of methionine increased ammonia-N excretion in all the groups, and decreased urea-N excretion in the animals fed the 80 g and 60 g protein/kg diet.

Table 5.13 Ammonia-N and urea-N excretion of rats fed diets of graded casein content supplemented with methionine to equalise dietary sulphur amino acid content-average of two days before starting treatments (μmol/day)

Dietary groups g protein/kg diet	NP+M 180+M	LP+M 80+M	VLP+M 60+M	one-way ANOVA p-value F ratio
Ammonia-N	851 ± 35^a	392 ± 16^b	403 ± 19^b	p=0.000 F=108.59
Urea-N	10187 ± 371^a	852 ± 36^b	699 ± 42^c	p=0.000 F=603.95

* Results are presented as mean ± standard error of the mean, n=12 per group. If significant effects due to diet were found. Means within each row having different letter superscripts following the number differ significantly.

Ammonia-N and urea-N excretion after starting treatments

a) Ammonia-N excretion

Ammonia-N excretion in the LP+M and the VLP+M groups, whether the animals were fed *ad libitum*, LPS treated or given low-dietary intake, was significantly lower than in the NP+M group (Table 5.14).

Comparing the animals fed *ad libitum,* LPS-treated rats and pair-fed controls, it can be seen that in the NP+M group there was a significant reduction in ammonia-N excretion after food restriction, but not after LPS treatment when compared to values from animals fed *ad libitum.* In the VLP+M group, ammonia-N excretion was reduced significantly after LPS treatment, but not after food restriction when compared to values from animals fed *ad libitum.*

Equalising the sulphur amino acid content of all dietary groups increased ammonia-N excretion compared with the previous experiment (Table 4.15, chapter 4). This increase in ammonia-N excretion, after methionine supplementation, was greater in the low-protein dietary groups (the LP+M and VLP+M groups).

b) Urea-N excretion

In animals fed *ad libitum*, pair-fed controls and LPS-treated animals, urea-N excretion was significantly reduced as dietary protein content was decreased (Table 5.14).

Comparing the LPS treated rats with pair-fed animals, it can be seen that the mean values increased in all dietary groups after LPS treatment compared with values for the pair-fed controls.

A comparison with the data in Table 4.15, chapter 4, shows that equalising the sulphur amino acid content in the diets containing graded levels of casein resulted in a reduction in urea-N excretion in all treatment groups fed the 80 g protein/kg diet and in LPS and *ad libitum*-fed animals given the 60 g protein diets compared with animals fed the same amount of casein but without methionine supplementation in the previous experiment. However, it increased urea-N excretion in all treatment groups given the NP+M when compared with animals fed the NP diet, without methionine supplementation in the previous experiment.

Table 5.14 Ammonia-N and urea-N excretion of rats fed diets of graded casein content supplemented with methionine to equalise dietary sulphur amino acid content, after starting treatments (µmol/day)

Dietary groups	NP+M	LP+M	VLP+M	two-way ANOVA	
g protein/kg diet	180+M	80+M	60+M	p-value	F ratio
Ammonia-N					
ad libitum	798 ± 20^{a}	333 ± 30^{b}	345 ± 20^{b}	D=0.000	F=129.73
pair-fed	550 ± 17^{a}*	245 ± 18^{b}	262 ± 38^{b}	T=0.000	F=12.2
LPS	705 ± 77^{a}	244 ± 36^{b}	212 ± 25^{b}*	I=0.055	F=2.66
Urea-N					
ad libitum	10713 ± 634^{a}	830 ± 50^{b}	644 ± 47^{c}	D=0.000	F=246.25
pair-fed	8571 ± 226^{a}	607 ± 42^{b}	512 ± 34^{b}	T=0.206	F=1.67
LPS	9668 ± 1563^{a}	1030 ± 96^{b}	690 ± 66^{c}	I=0.417	F=1.02

-Results are presented as mean ± standard error of the mean, n=4 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

5.4.6 Relationship between parameters measured in the study

The ratio of glutathione to protein per gram liver after starting treatments

Dietary protein supplemented with methionine, food intake and LPS treatment influenced the ratio of GSH to protein in liver (Table 5.15).

In the animals fed *ad libitum*, the ratio of GSH to protein was significantly higher in rats fed the VLP+M diet compared with other groups fed the NP+M and LP+M diets. In all the groups, restricting food intake by pair feeding caused a significant reduction in the ratio compared with the *ad libitum* groups. In the pair-fed controls, the ratio was higher in the VLP+M group compared with NP+M group. In the LPS-treated animals, there was an increase in the ratio as dietary protein content was decreased. Due to treatments, the ratio was significantly higher in all the groups after LPS treatment compared with the pair-fed animals.

Equalising the sulphur amino acid content in all dietary groups dramatically increased the liver ratio of glutathione to protein in animals fed the 80 g and 60 g protein/kg diet compared with the previous experiment in which animals fed the same diets but without methionine supplementation. This increase in the ratio was greater in the *ad libitum* and LPS-treated animals.

Table 5.15 The ratio of hepatic glutathione to hepatic protein and to inorganic sulphate excretion of rats fed diets of graded casein content supplemented with methionine to equalise dietary sulphur amino acid content, after starting treatments

Dietary groups g protein/kg diet	NP+M 180+M	LP+M 80+M	VLP+M 60+M	two-way ANOVA p-value F ratio
GSH/ protein (nmol/mg)				
ad libitum	40.3 ± 1.2 [a]	40.3 ± 2.8 [a]	63.2 ± 5.0 [b]	D=0.000 F=20.27
pair-fed	16.2 ± 2.4 [a] *	20.6 ± 2.8 [a b] *	30.3 ± 4.5 [b] *	T=0.000 F=61.82
LPS	42.3 ± 1.3 [a] ♠	50.0 ± 1.5 [b] ♠ *	53.9 ± 2.3 [b] ♠	I=0.053 F=2.57
GSH/SO_4 (nmol per liver /µmol per day)				
ad libitum	117 ± 4 [a]	103 ± 7 [a]	134 ± 12 [a]	D=0.054 F= 3.25
pair-fed	44 ± 6 [a] *	59 ± 15 [a b]	80 ± 9 [b] *	T=0.000 F= 46.87
LPS	120 ± 8 [a] ♠	152 ± 11 [a] ♠ *	128 ± 4 [a] ♠	I=0.045 F=2.81

-Results are presented as mean ± standard error of the mean. For the ratio of hepatic glutathione to hepatic protein, n=4 per treatment group for animals fed the NP+M diet, and n=6 per treatment group for animals fed the LP+M and the VLP+M diets. For the ratio of hepatic glutathione to inorganic sulphate excretion, n=4 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

The ratio of total hepatic glutathione to inorganic sulphate excretion in urine after starting treatments

There was an increase in the ratio of total hepatic GSH to SO_4 excreted in urine in all dietary groups after LPS treatment (Table 5.15).

In the *ad libitum* and in LPS-treated animals, no significant difference in the ratio of total hepatic GSH to SO_4 excretion in urine was found among the different dietary groups. In the pair-fed animals, the ratio was significantly higher in the VLP+M group compared with the NP+M group. Dietary protein and sulphur amino acid content had no effect upon the ratio, however, it was reduced by restricting food intake.

A comparison with the data in Table 4.16, chapter 4, addition of methionine increased the ratio of total hepatic GSH to SO_4 excretion in the 80 g and 60 g protein/kg diet in rats given LPS or fed *ad libitum*. When food was restricted, the addition of methionine resulted in a reduction in ratio. On the 180 g protein/kg diet, the addition of methionine reduced the ratio in all treatment groups.

The ratio of ammonia-N and urea-N excretion to N intake-average of two days before starting treatments

The ratio of ammonia-N to N intake was not affected by the reduction in dietary protein concentration from 180 g to 80 g protein/kg diet, but was significantly higher in the VLP+M group compared with other dietary groups. The ratio of urea-N excretion to N intake was markedly affected by the reduction in dietary protein concentration (Table 5.16). The ratio was significantly lower in the LP+M and VLP+M groups compared with the N+M group.

In all dietary groups, addition of methionine increased the ratio of ammonia-N to N intake by 66%, 190%, and 258% respectively for the NP+M, LP+M and VLP+M groups, when compared with the previous study. For the ratio of urea-N to N intake, addition of methionine decreased the ratio by 42% and 38% respectively for the LP+M and VLP+M groups, and increased the ratio by 22% for the NP+M group, when compared with the previous study (Table 4.17, chapter 4).

Table 5.16 The ratio of ammonia-N and urea-N excretion to N intake of rats fed diets of graded casein content supplemented with methionine to equalise dietary sulphur amino acid content-average of two days before starting treatments (mg/mg)

Dietary groups g protein/kg diet	NP+M 180+M	LP+M 80+M	VLP+M 60+M	one-way ANOVA p-value	F ratio
Ammonia-N/N intake	0.028 ± 0.001^{a}	0.028 ± 0.002^{a}	0.037 ± 0.002^{b}	p=0.000	F=15.11
Urea-N/N intake	0.339 ± 0.015^{a}	0.062 ± 0.003^{b}	0.065 ± 0.004^{b}	p=0.000	F=296.1

* Results are presented as mean ± standard error of the mean, n=12 per group. Means within each row having different letter superscripts following the number differ significantly.

The ratio of ammonia-N and urea-N excretion to N intake after starting treatments

a) The ratio of ammonia-N excretion to N intake

Due to diet, an increase in the ratio occurred in the VLP+M group compared to other dietary groups. This was statistically significant in the pair-fed controls when comparing the VLP+M group with the NP+M group. In the LPS-treated animals and in animals fed *ad libitum*, the mean values were higher in the VLP+M group compared with other dietary groups, but this increase in the ratio was not statistically significant.

No difference in the ratio of ammonia-N excretion to N intake was found between the groups due to treatments (Table 5.17).

Addition of methionine increased the ratio of ammonia-N excretion to N intake in all treatment groups compared with the previous experiment (Table 4.18, chapter 4). In the previous experiment, the ratio of ammonia-N to N intake reduced as dietary protein content was decreased, but to less extent than the ratio of urea-N excretion to N intake. In the present study, the ratio of ammonia-N excretion to N intake did not fall when the protein content in the diet decreased. In the present study as in the previous study, ammonia-N excretion was not affected by restricted feeding or exposure to the inflammatory influence of LPS.

Table 5.17 The ratio of ammonia-N and urea-N excretion to N intake of rats fed diets of graded casein content supplemented with methionine to equalise dietary sulphur amino acid content, after starting treatments (mg/mg)

Dietary groups g protein/kg diet	NP+M 180+M	LP+M 80+M	VLP+M 60+M	two-way ANOVA p-value	 F ratio
Ammonia-N/N intake					
ad libitum	0.025 ± 0.003^{a}	0.026 ± 0.004^{a}	0.028 ± 0.001^{a}	D=0.004	F=6.66
pair-fed	0.022 ± 0.002^{a}	0.027 ± 0.002^{ab}	0.039 ± 0.004^{b}	T=0.341	F=1.12
LPS	0.028 ± 0.001^{a}	0.026 ± 0.001^{a}	0.031 ± 0.004^{a}	I=0.098	F=2.18
Urea-N/N intake					
ad libitum	0.333 ± 0.017^{a}	0.065 ± 0.011^{b}	0.052 ± 0.001^{b}	D=0.000	F=136.04
pair-fed	0.351 ± 0.035^{a}	0.068 ± 0.008^{b}	0.081 ± 0.015^{b}	T= 0.037	F=3.72
LPS	0.374 ± 0.038^{a}	0.122 ± 0.031^{b}	$0.106 \pm 0.023^{b*}$	I=0.949	F=0.18

-Results are presented as mean ± standard error of the mean, n=4 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair fed or LPS treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

b) The ratio of urea-N excretion to N intake

The ratio of urea-N excretion to N intake was affected by dietary protein content supplemented with methionine and the inflammatory influence of exposure to LPS. A restriction in food intake had no effect in the ratio (Table 5.17).

In the *ad libitum*, LPS treated and pair-fed groups, the ratio of urea-N excretion to N intake was significantly lower in the LP+M and VLP+M groups compared with the NP+M group.

Due to treatments, the mean values was higher in all the groups after LPS treatment compared with the *ad libitum* and pair-fed animals, and this increase was considerable in rats fed the LP+M and VLP+M diets, reaching statistical significance ($p<0.05$) in the VLP+M group.

Addition of methionine reduced the ratio of urea-N to N intake in the *ad libitum*, LPS treated and pair-fed animals given the 80 g protein/kg diet. In animals fed the 60 g protein/kg diet, addition of methionine reduced the ratio in the LPS-treated rats and in *ad libitum*-fed animals (see Table 4.18, chapter 4).

The ratio of inorganic sulphate excretion to ammonia-N and urea-N excretion-average of two days before starting treatments

a) The ratio of inorganic sulphate excretion to ammonia-N excretion

The ratio of SO_4 to ammonia-N excretion was significantly higher in the LP+M and the VLP+M groups compared with the NP+M group. This increase in the ratio was mainly due to the significant reduction in NH_3-N excretion that was found in the LP+M and the VLP+M groups. No difference was found in the ratio between the LP+M and VLP+M groups (Table 5.18).

Paradoxically, after addition of methionine, the ratio of SO_4 excretion to ammonia-N excretion was reduced in animals fed the low-protein diets (80 g and 60 g protein/kg diet) compared with the previous study, in which animals fed the same diets but without methionine supplementation (Table 4.19, chapter 4).

Table 5.18 The ratio of inorganic sulphate excretion to ammonia-N and urea-N excretion of rats fed diets of graded casein content supplemented with methionine to equalise dietary sulphur amino acid content-average of two days before starting treatments (μmol/μmol)

Dietary groups g protein/kg diet	NP+M 180+M	LP+M 80+M	VLP+M 60+M	one-way ANOVA p-value F ratio
SO_4/NH_3-N	0.38 ± 0.01^a	0.90 ± 0.02^b	0.97 ± 0.04^b	p=0.000 F=162.82
SO_4/urea-N	0.032 ± 0.001^a	0.422 ± 0.023^b	0.574 ± 0.043^c	p=0.000 F=99.17

* Results are presented as mean ± standard error of the mean, n=12 per group. Means within each row having different letter superscripts following the number differ significantly.

b) The ratio of inorganic sulphate excretion to urea-N excretion

The ratio of SO_4 excretion to urea-N excretion increased significantly as dietary protein content in the diet was decreased (Table 5.18). The increase in the

ratio was mainly due to the significant reduction in urea-N excretion that was found in the LP+M and the VLP+M groups compared with the NP+M group

After addition of methionine, the ratio of SO_4 excretion to urea-N excretion increased in all dietary groups compared with the previous study in which animals fed the same diets but without added methionine (Table 4.19, chapter 4). The magnitude of the increase in the ratio was much greater in the LP+M and VLP+M groups than in the NP+M group.

The ratio of inorganic sulphate excretion to ammonia-N and urea-N excretion after starting treatments

a) The ratio of inorganic sulphate excretion to ammonia-N excretion

The ratio of SO_4 excretion to ammonia-N excretion can be seen in Table 5.19. In the animals fed *ad libitum*, LPS-treated rats, and pair-fed controls, the ratio of SO_4 to ammonia-N excretion was significantly higher in the LP+M and VLP+M groups compared with the NP+M group. No difference was found in the ratio between the LP+M and VLP+M groups.

Due to treatments, in the VLP+M group, the magnitude of the ratio was higher in the LPS-treated animals than in the corresponding pair-fed and *ad libitum*-fed animals, but this difference was not statistically significant. Otherwise, neither LPS nor restriction in food intake caused an alteration in the ratio.

Table 5.19 The ratio of inorganic sulphate excretion to ammonia-N and urea-N excretion of rats fed diets of graded casein content supplemented with methionine to equalise dietary sulphur amino acid content, after starting treatments (μmol/μmol)

Dietary groups g protein/kg diet	NP+M 180+M	LP+M 80+M	VLP+M 60+M	two-way ANOVA p-value	 F ratio
SO_4/NH_3-N					
ad libitum	0.40 ± 0.02 [a]	0.92 ± 0.12 [b]	1.12 ± 0.04 [b]	D=0.000	F=69.9
pair-fed	0.46 ± 0.01 [a]	1.02 ± 0.06 [b]	1.09 ± 0.13 [b]	T=0.336	F=1.14
LPS	0.44 ± 0.04 [a]	1.06 ± 0.12 [b]	1.34 ± 0.10 [b]	I= 0.482	F=0.89
SO_4/urea-N					
ad libitum	0.030 ± 0.0003 [a]	0.419 ±0.022 [b]	0.603 ± 0.037 [c]	D=0.000	F=215.86
pair-fed	0.030 ± 0.001 [a]	0.414 ± 0.042 [b]	0.535 ± 0.024 [b]	T=0.000	F=13.48
LPS	0.034 ± 0.005 [a]	0.251 ± 0.036 [b]*♠	0.417 ± 0.048 [c]*	I=0.012	F= 3.91

-Results are presented as mean ± standard error of the mean, n=4 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

b) The ratio of inorganic sulphate excretion to urea-N excretion

The ratio of SO_4 to urea-N excretion was influenced by dietary protein intake supplemented with methionine. In the LP+M and the VLP+M groups, the ratio was also influenced by LPS treatment (Table 5.19).

The ratio of SO_4 excretion to urea-N excretion was increased significantly in the *ad libitum* fed and in LPS-treated animals as dietary protein content was decreased. In the pair-fed animals there was a trend for an increase in the ratio as dietary protein content was decreased from 180 g to 80 and 60 g/kg diet.

In the LP+M group, the ratio in the LPS-treated animals was significantly lower than in the *ad libitum* and pair-fed animals. In the VLP+M group, the LPS-treated animals had a lower ratio than the animals fed *ad libitum*. In the low-protein groups (the LP+M and VLP+M groups), the ratio in the LPS-treated animals was also lower than in the pair-fed controls, but only achieved statistical significance in the LP+M group.

After addition of methionine, the ratio of SO_4 excretion to urea-N excretion was increased in all treatment groups compared with the previous study in which animals fed the same diets, but without methionine supplementation (Table 4.20,

chapter 4). The magnitude of the increase in the ratio was greater in animals fed the 80 g and 60 g protein diets.

5.4.7 Number of neutrophils in lung

Unlike the previous study, no significant difference in the number of neutrophils in lung was found between the groups due to diet or treatments (see Table 5.20 & Table 4.21, chapter 4). Dietary supplementation with methionine appeared to prevent the fall in neutrophil numbers which occurred in the *ad libitum* and in pair-fed animals, in the previous study, when the low-protein diets without methionine supplementation were consumed, and to suppresses the increase in neutrophil numbers which happened when animals, on the low-protein diets without methionine supplementation, received an inflammatory challenge.

Table 5.20 Number of neutrophils in lung of of rats fed diets of graded casein content supplemented with methionine to equalise dietary sulphur amino acid content (number of neutrophils in lung section/number of intersection points occupied by lung section) × 100

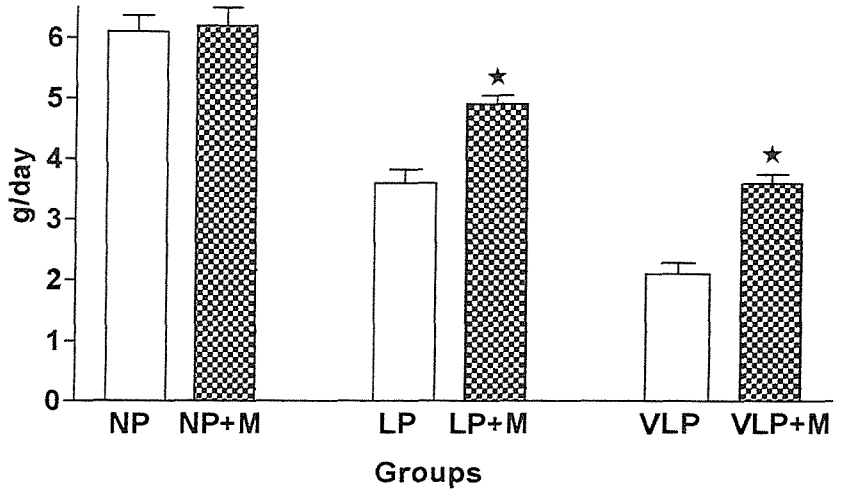
Dietary groups	NP+M	LP+M	VLP+M	two-way ANOVA	
g protein/kg diet	180+M	80+M	60+M	p-value	F ratio
ad libitum	15.6 ± 0.8 [a]	14.7 ± 1.3 [a]	13.9 ± 0.9 [a]	D=0.368	F=1.04
pair-fed	14.3 ± 1.5 [a]	12.8 ± 0.8 [a]	13.1 ± 0.9 [a]	T=0.057	F=3.18
LPS	15.7 ± 0.6 [a]	14.5 ± 1.1 [a]	16.3 ± 1.0 [a]	I=0.726	F=0.51

-Results are presented as mean ± standard error of the mean, n=4 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

5.5 Discussion

Addition of methionine to the low-protein diets enhanced the growth process, but had no effect on growth when it was added to the normal-protein diet (Figure 5-2). The data clearly indicates that methionine was limiting for growth in animals fed the low-protein diets, but not in animals fed the normal-protein diet.

Figure 5-2 Average daily weight gain of animals fed graded levels of casein with and without methionine supplementation



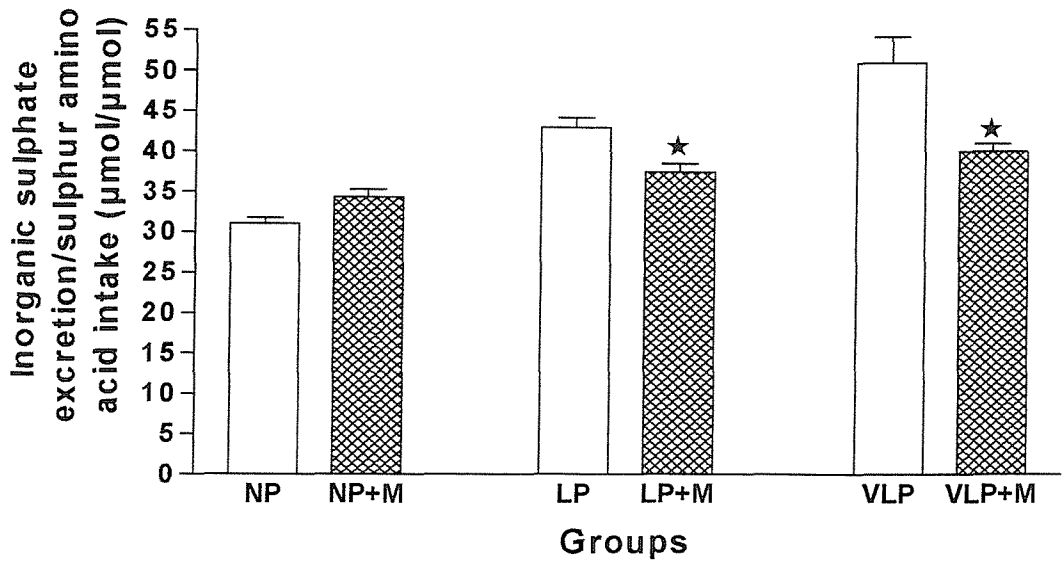
Fed diets of graded levels of casein.

Fed diets of graded levels of casein supplemented with methionine.

Results are means ± SE. * Significantly different from animals fed the same diet, but without methionine supplementation (n 18).

Addition of methionine may have improved the metabolic utilisation of sulphur amino acids in the low-protein and very low-protein dietary groups as indicated by the reduction in SO_4 excretion, as a percentage of sulphur amino acid intake, compared with the previous experiment (Table 4.12, chapter 4; Table 5.11 & Figure 5-3). The effect was greatest in the VLP group, where SO_4 excretion, as a percentage of sulphur amino acid intake, fell from 51% to 40%. In the LP group a more modest effect was achieved with a fall from 43% to 37%. Improvement in the ratio of urea-N to N intake also occurred as discussed below.

Figure 5-3 Inorganic sulphate excretion as a percentage of sulphur amino acid intake in animals fed graded levels of casein with and without sulphur amino acid supplementation



Results are means ± SE. ⋆ significantly different from animals fed the same diet, but without methionine supplementation (n 12).

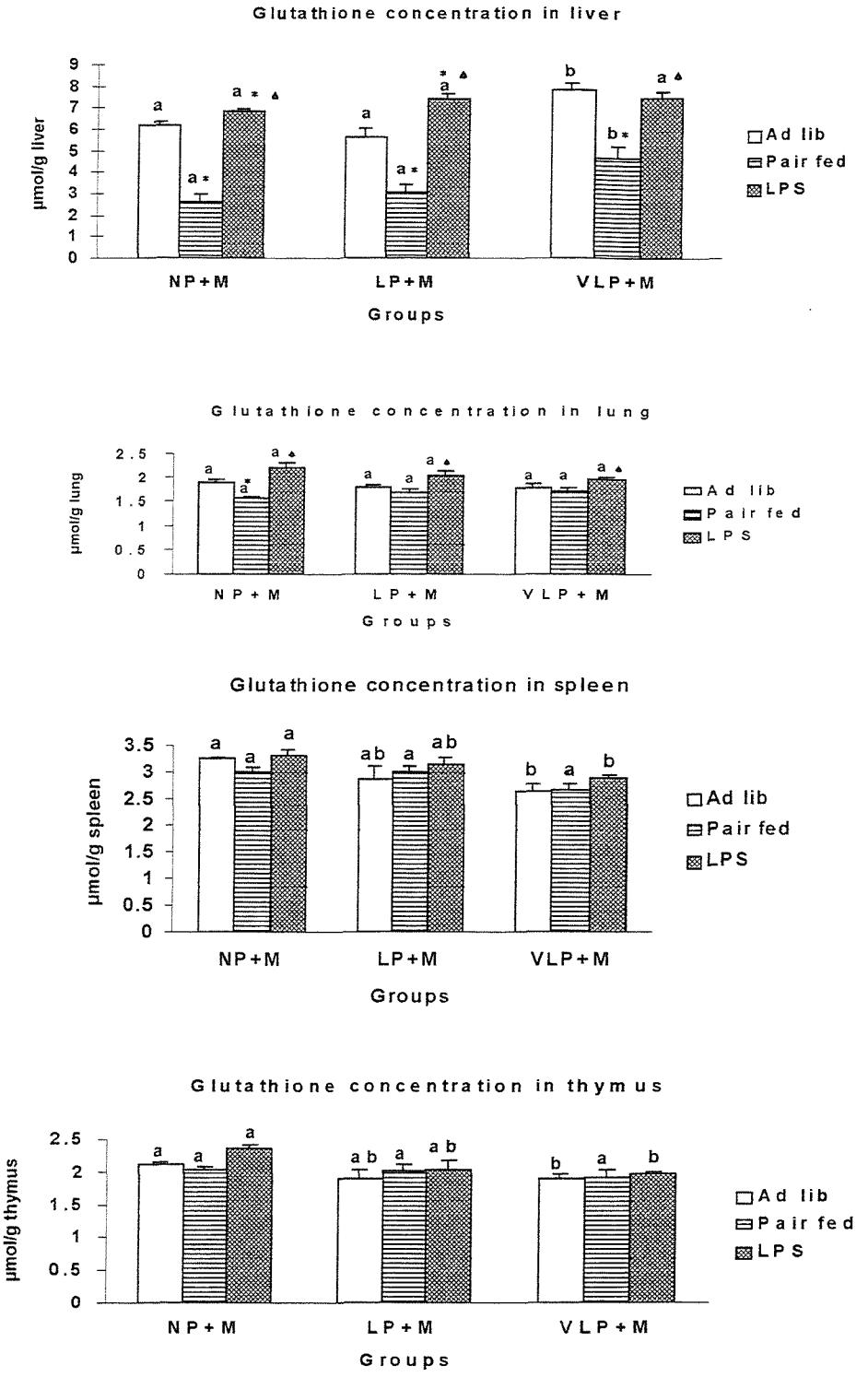
The improved utilisation of protein for growth, achieved by addition of methionine to the low-protein diets, resulted in differential effects upon growth of individual organs. The relative liver weights of animals fed the LP+M and the VLP+M diets were greater than in animals fed the same diets, but without methionine supplementation in the previous study. However, there was a lack of a similar effect on the relative lung, spleen, and thymus weight. In particular the depressed spleen weight, which occurred in animals fed the low-protein diets, failed to respond to methionine supplementation.

Methionine supplementation exerted major effects on tissue composition. Addition of this amino acid to the low-protein diets increased glutathione concentrations in various organs compared with animals fed the same diets, but without methionine supplementation, in the previous experiment. Furthermore, the addition of methionine to the VLP diet prevented the fall in GSH concentration that occurred in lung, spleen, and thymus in response to LPS. After addition of

methionine, the magnitude of the increase in glutathione concentration in various organs was greatest in liver (see Table 4.7, chapter 4; Figure 5-4 & Figure 5-5). This effect indicates that liver is the most sensitive organ in responding to dietary manipulation of the substrate, cysteine, for GSH synthesis.

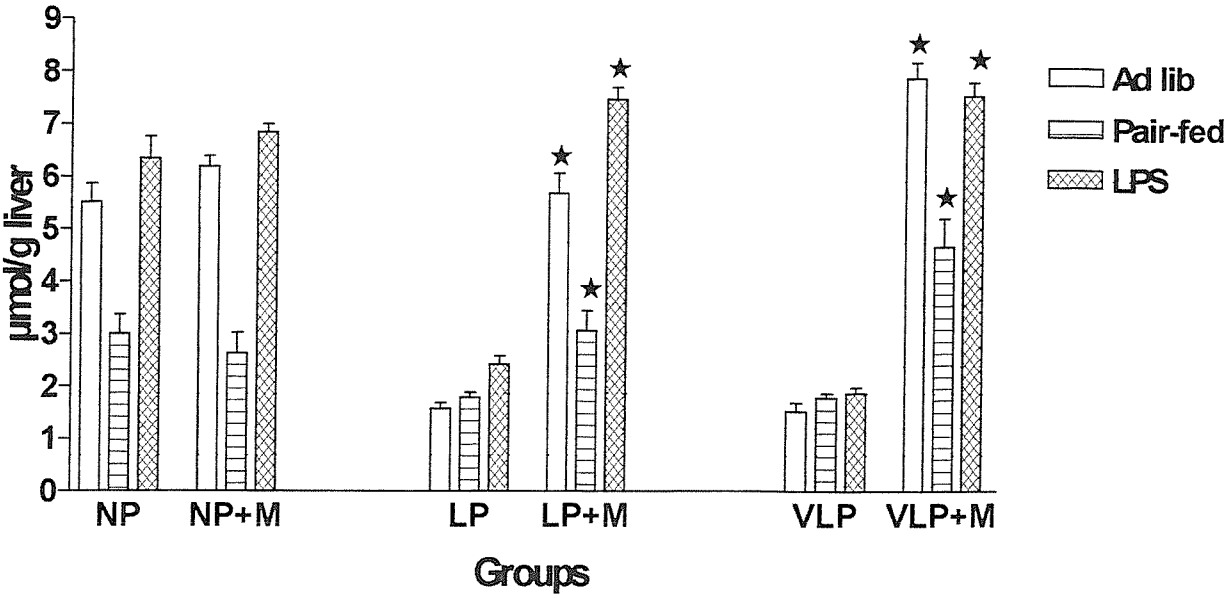
Paradoxically, hepatic GSH concentration in animals fed the VLP+M diet *ad libitum* was significantly higher than in other animals fed the LP+M and the NP+M diets *ad libitum*, despite a similarity in the amount of sulphur amino acid intake between the groups. A low utilisation of methionine and cysteine for protein synthesis, because of severe limitations in other amino acids, may have increased cysteine availability, and could explain the higher GSH concentration found in the VLP+M group (see Figure 5-6). For this situation to occur, however, there would have to be adequate availability of glycine and glutamic acid. Enhanced deamination of amino acids not used for growth could provide the necessary substrate for synthesis of the latter molecule.

Figure 5-4 Glutathione concentration in liver, lung, spleen, and thymus



-Results are means ± SE. N=4 per treatment group for animals fed the NP+M diet, and n=6 per treatment group for animals fed the LP+M and the VLP+M diets. Means within each treatment group having different letter superscripts differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

Figure 5-5 Hepatic glutathione concentration in animals fed graded levels of casein with and without methionine supplement

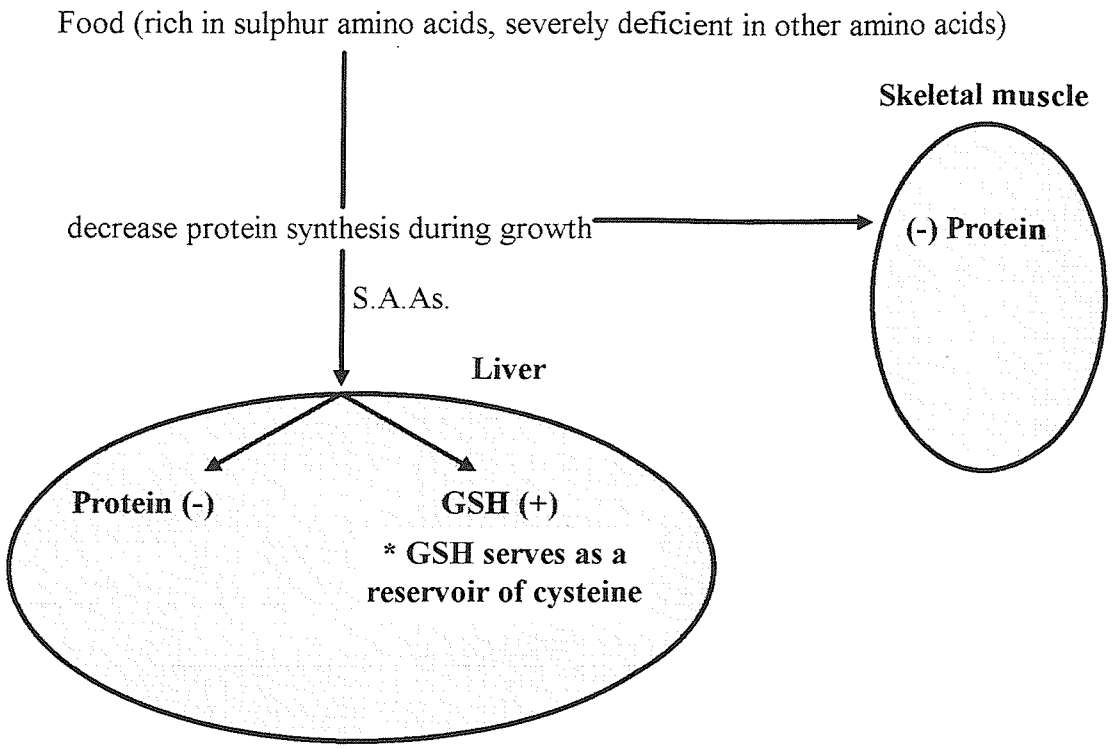


Results are means ± SE. N=6 per treatment group for animals fed the NP, LP, VLP, LP+M, VLP+M diets, and n=4 per treatment group for animals fed the NP+M diet. ⋆Significantly different from animals having the same diet and treatment, but without methionine supplementation.

Other studies in unstressed animals have shown the sensitivity of hepatic GSH to sulphur amino acid supply. Bauman *et al.* (1988b) have shown that rats fed 75 g protein/kg diet, had a lower hepatic GSH than rats fed 150 g protein/kg diet, using casein as a source of protein. However, when the 75 g protein/kg diet was supplemented with L-2-oxothiazalidine-4-carboxylate (OTC), a stable derivative of cysteine to be isosulphurous with the 150 g protein/kg diet, hepatic GSH concentration in animals fed the 75 g protein + OTC/kg diet was not different from that in rats fed the 150 g protein/kg diet. This was consistent with our study in which hepatic GSH concentration in animals fed the LP+M diet was similar to that in animals fed the NP+M diet, despite the differences in total protein intake. However, Taylor *et al.* (1992) found that liver GSH concentration in rats fed 0.5 g protein + OTC/kg diet was higher than in rats fed 150 g protein/kg diet, despite a similarity in the amount of sulphur amino acids consumed by the groups. Although the protein

deficiency imposed by the dietary regimen of Taylor *et al.* (1992) was more severe than in the present study, the same combination of effects (improvement of the growth potential of a low-protein diet combined with supra normal hepatic GSH content) can be seen. In lung, GSH concentration increased by 47% after the 0.5 g protein/kg diet was supplemented with OTC compared with the 0.5 g protein/kg diet, but without OTC supplementation. However, in Taylor's study, OTC supplementation of the 0.5 g protein/kg diet did not increase lung GSH concentration to the same level found in the 150 g protein/kg diet. In mice, Adachi *et al.* (1992) reported a lower hepatic efflux rate of GSH in animals fed a low-protein diet compared with animals fed a normal-protein diet. In the present study, although animals fed the 60 g protein/kg diet, supplemented with methionine, were able to significantly increase hepatic GSH concentration compared with animals fed the NP+M diet, and lung GSH concentration was similar between the two dietary groups, spleen and thymus GSH concentrations were lower in animals fed the VLP+M diet compared with animals fed the NP+M diet. The results of the Adachi *et al.*'s study (1992) suggest that another possibility for the increase liver GSH concentration in rats fed the very low-protein diet supplemented with methionine might be a reduction in the efflux rate of hepatic GSH.

Figure 5-6 The partitioning of sulphur amino acid in animals fed a diet severely deficient in protein but supplemented with L-methionine



S.A.As.: Sulphur amino acids.
(-): Relatively low utilisation of S.A.As. for protein synthesis.
(+): Relatively high utilisation of S.A.As. for GSH synthesis.

It could be concluded from the result of hepatic GSH concentration that when animals were fed the VLP+M diet *ad libitum*, hepatic GSH production increased above that of the other groups. However, when an inflammatory stress was given, production in the VLP+M group was no longer greater than in the LP+M and NP+M group. What explanations are possible for this phenomenon?

In the animal model described in this thesis, two major anabolic processes compete for cysteine during growth and inflammation, namely, protein synthesis and GSH synthesis. Protein synthesis requires all amino acids, essential (from the diet), non-essential (synthesised by the body) and semi-essential (synthesised from essential amino acids; e.g. methionine → cysteine). On the other hand, GSH synthesis requires only cysteine, glycine and glutamate. When rats were fed a diet severely deficient in protein, but rich in methionine (e.g. the animals fed the VLP+M

diet), shortage of amino acids restricts the amount of protein synthesis, therefore, more sulphur amino acids were available for the biosynthetic pathway of GSH, provided that there is enough glycine and glutamate. Consequently, GSH synthesis was increased in animals fed the very low-protein diet *ad libitum* when compared with other dietary groups. However, during inflammation, the wide range of amino acids released from muscle relieves the shortage of amino acids that may have restricted protein synthesis. Consequently, cysteine can be used in protein synthesis and hence GSH, relatively, was not increased in animals fed the VLP+M diet when compared with other dietary groups. In other words, during the inflammatory response, the rats' muscle protein is able to supply the amino acids, which were being restricted by feeding a diet severely deficient in protein content.

In addition to synthesis, the rate of recycling and utilisation of GSH and GSSG will have an effect on tissue GSH content. The relative activities of GPx and GR will influence these processes. In the present study, the LPS-treated and *ad libitum*-fed animals given the 60 g protein/kg diet (the VLP+M diet) had a higher GPx activity compared with the animals given the NP+M diet. However, when compared with results from the previous study, the addition of methionine to the NP+M, LP+M and VLP+M diets resulted in a fall in liver GPx activity (see Table 4.8, chapter 4 & Table 5.7). The reduction in hepatic GPx activity in the *ad libitum* and in LPS-treated animals in the LP+M and VLP+M groups compared with animals fed the same level of protein, but without methionine supplementation in the previous experiment, could contribute to the dramatic increase in hepatic GSH concentration after addition of methionine. Furthermore, this reduction in hepatic GPx activity could be partly explained by Kleiber's theory (1944 & 1961) which stated that ingestion of an imbalance diet, in which a single nutrient was limited, results in a decreased efficiency of energy utilisation, and that addition of that nutrient to the diet increases the efficiency of energy utilisation. The energy efficiency of the low-protein dietary groups supplemented with methionine in the present study was increased compared with the low-protein dietary groups, without methionine supplementation in the previous study. This indicates that animals fed

the LP+M and VLP+M diets deposited a greater proportion of the energy content of the diet into tissue growth and were thus required to oxidised a smaller proportion of food energy in the process of thermogenesis than animals fed the low-protein diets, without methionine supplementation. Accordingly, the LP+M and VLP+M groups would have a lower resting metabolic rate and would be produce less oxygen free radicals than animals fed the low-protein diets, but without methionine supplementation. On the other hand, Kleiber's theory cannot explain the reduction in GPx activity that was found after addition of methionine to the normal-protein diet, since the energy efficiency was slightly reduced after addition of methionine, and the average daily weight gain was similar between the NP and the NP+M groups. The decrease in GPx activity could explain the increase (not statistically significant) in hepatic GSH concentration after addition of methionine to the normal-protein diet compared with animals fed the normal-protein diet, without methionine supplementation.

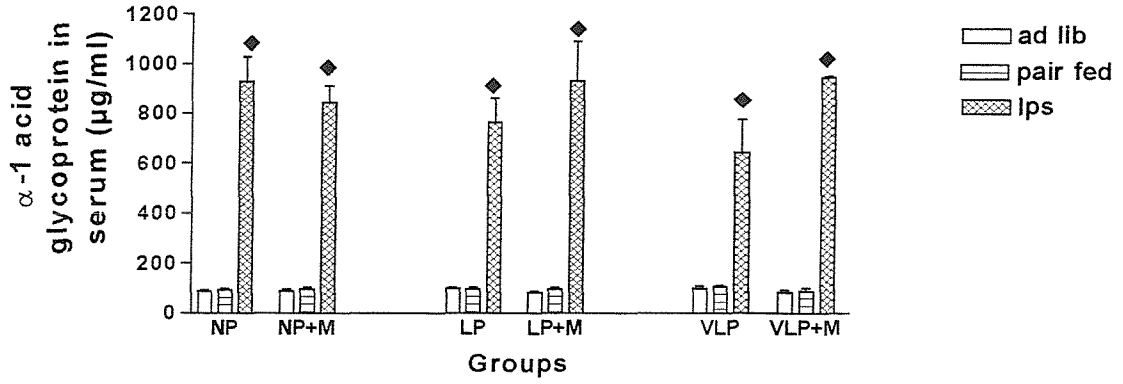
GR activity in liver was significantly higher in the VLP+M group compared with the NP+M and LP+M groups, in the *ad libitum* and in LPS-treated animals. However, addition of methionine to all dietary groups had no effect on the activity of GR compared with values in the previous experiment (Table 4.9, chapter 4 & Table 5.8).

In addition to hepatic GSH metabolism; protein metabolism in the organ was profoundly affected by supplementation of the low-protein diets with methionine. Methionine supplementation appears to increase liver protein, as a proportion of body weight, when values are compared with those of animals receiving the same level of protein, but without methionine supplementation (Table 4.10, chapter 4 & Table 5.9). Furthermore, addition of methionine to the 80 g and 60 g protein/kg diet, allowed relative liver protein to increase in response to LPS.

What was true for liver protein was also partly the case for individual export proteins. In particular, the acute phase protein, α-1 acid glycoprotein. Comparison of the present and previous studies (Figure 5-7), showed that addition of methionine to the low-protein diets did not influence α-1 acid glycoprotein concentration in

animals fed *ad libitum* and in pair-fed controls. However, in the endotoxin-treated animals, a stimulatory effect of addition of methionine to the low-protein diets was apparent. However, this increase was not statistically significant. Addition of methionine to all diets had no effect on the serum concentration of albumin (see Table 4.11, chapter 4 & Table 5.10).

Figure 5-7 Serum α-1 acid glycoprotein in animals fed graded levels of casein without and with methionine supplementation



- Results are means ± SE. N=6 per treatment group for animals fed the NP, LP, VLP, LP+M, VLP+M diets, and n=4 per treatment group for animals fed the NP+M diet.

◆ Indicates a significant difference between LPS-treated animals and the corresponding *ad libitum* and pair-fed animals

Equalising the sulphur amino acid content of the diets of graded casein content dramatically changes the relative amounts of many of the major end products of amino acid supplementation. The ratio of GSH to protein in the liver of animals fed the 80 g and 60 g protein/kg diet was increased when compared with the results from previous experiment in which diets were not supplemented with methionine. The increase in the ratio was greatest in the *ad libitum* and in LPS-treated animals (see Table 4.16; chapter 4 & Table 5.15). This increase in the ratio could benefit the body when it is exposed to an inflammatory stimulus. However, it could also be an indication of the inefficiency of utilising sulphur amino acids for building tissue "growth", because of limited availability of other amino acids. Glutathione may be serving as a reservoir for cysteine to prevent toxicity of this amino acid. As a result

of this adaptation, the animals would appear to become more efficient in conserving sulphur amino acid in the presence of methionine supplementation than in its absence.

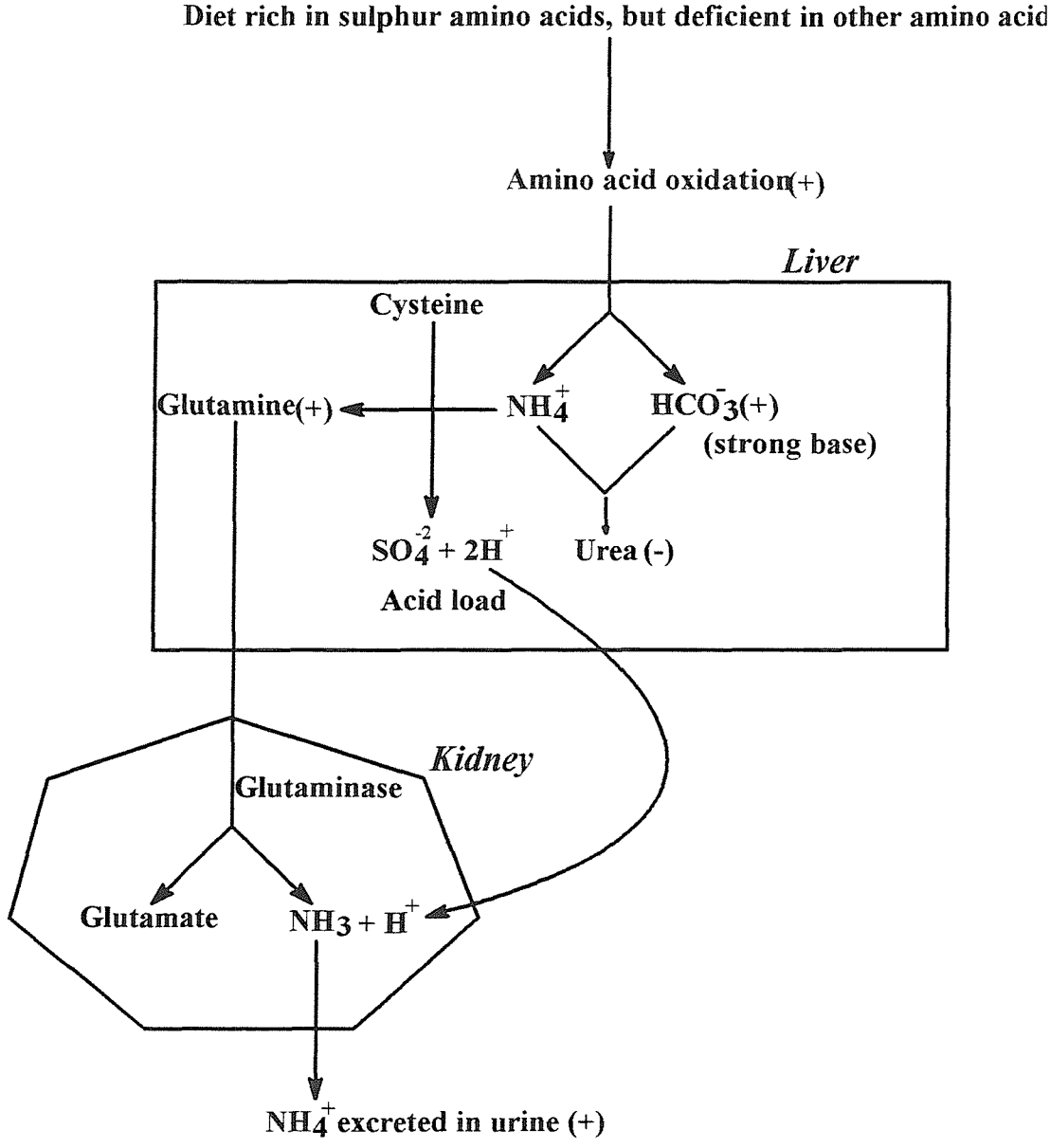
Although urinary urea and ammonia are both nitrogenous end products of amino acid metabolism, it is clear from the results in chapter 4 and the present chapter that both metabolites are affected in different ways by dietary protein and sulphur amino acid intake and inflammation. Part of the reason for the difference lies with NH_3 having a key role in acid base balance of the animals as well as in N metabolism.

The oxidation of amino acids produces HCO_3^- and NH_4^+. Ammonia detoxification, then, can be achieved by synthesising urea or glutamine in the liver. Urea synthesis takes place in periportal hepatocytes, while glutamine synthesis takes place in perivenous hepatocytes. In acidosis, when there is a high acid load, urea synthesis decreases, while glutamine synthesis increases. Consequently, HCO_3^- is retained in the liver to assist pH homeostasis. The decreased production of urea is accompanied with an increase in the transportation of ammonia from periportal to perivenous cells, were the enzyme glutamine synthetase is found. Glutamine synthesised by perivenous hepatocytes is transported to the kidney, were the renal enzyme glutaminase is found. The enzyme hydrolyses the amide nitrogen of glutamine producing glutamate and free ammonia. Ammonia (NH_3) reacts with H^+ to form ammonium ion (NH_4^+), which is excreted in the urine. (for review see Haussinger, 1990).

The catabolism of cysteine produces one mole of SO_4 and two moles of H^+ per mole of cysteine metabolised (Bella & Stipanuk, 1995). SO_4 excretion in the present experiment increased significantly in all dietary groups compared with the previous experiment. This strongly suggests that the generation of H^+ may have been increased accordingly. To alleviate the acidosis which would occur from the increased generation of H^+, the recruitment of ammonium ions to urea biosynthesis has to be decreased, and hepatic glutamine synthesis has to be increased (see Figure 5-8). This was consistent with the increase in ammonia-N excretion and in the ratio

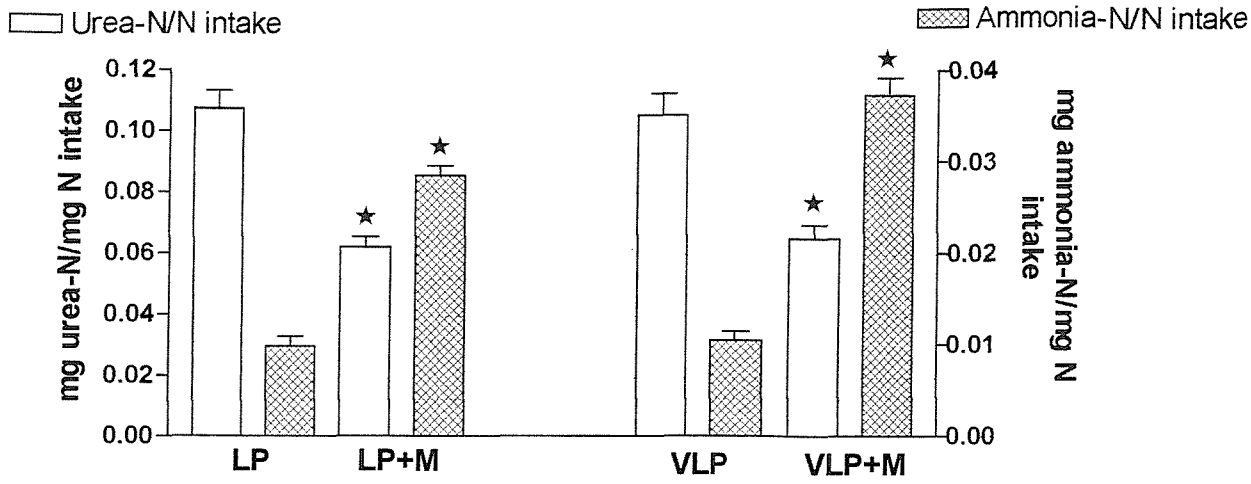
of ammonia-N excretion to N intake, in the reduction in urea-N excretion and in the ratio of urea-N excretion to N intake found in animals fed the low-protein diets supplemented with methionine (the LP+M and VLP+M diets) compared with animals fed the same diets, but without methionine supplementation (see Table 4.14, chapter 4; Table 5.13 & Figure 5-9) .

Figure 5-8 The partitioning of ammonia between urea and glutamine synthesis in rats fed a diet rich in sulphur amino acids



(+): Increased.
(-): Decreased.

Figure 5-9 The ratio of urea-N and ammonia-N excretion to N intake in animals fed graded casein with and without added methionine



- Results are means ± SE (n=12).
-Urea-N excretion/N intake: ⋆ Significantly different from animals fed the same diet, but without methionine supplementation.;
-Ammonia-N excretion/N intake: ⋆ Significantly different from animals fed the same diet, but without methionine supplementation.

These findings agree with studies by Bella & Stipanuk (1995), in which total ammonia excretion and ammonia excretion, as a percentage of total urinary nitrogen excretion, in animals fed a low-protein diet supplemented with 1.5 g methionine/kg diet, were 186 μmol/mg creatinine and 5.2% respectively, while in animals fed the same level of protein supplemented with 24g methionine/kg diet, ammonia excretion and ammonia excretion, as a percentage of total urinary nitrogen excretion, were increased significantly to 691μmol/mg creatinine and 18.8% respectively. Furthermore, the present study showed a decrease in SO_4 excretion, as a percentage of sulphur amino acid intake, in animals fed the low-protein diets supplemented with methionine compared with animals fed the same level of protein, but without methionine supplementation in the previous chapter (Figure 5-3). This change in response could be explained by the increase production of taurine at the expense of SO_4 excretion. This was consistent with the findings of Bagley & Stipanuk (1995) who found that hepatocytes incubated with 0.2 mmol/L [^{35}S] cysteine, [^{35}S] taurine

production, as a percentage of total [^{35}S] cysteine catabolism, by hepatocytes isolated from rats fed a low-protein diet (10% casein diet, supplemented with 10 g L-methionine or 8 g L-cysteine/kg diet) was higher than in hepatocyes isolated from rats fed the same diet, but without sulphur amino acid supplementation. Taurine production, as a percentage of [^{35}S] cysteine catabolism, was 61% and 49% for rats given methionine and cysteine supplemented diets respectively, compared with only 22% for rats given the control diet. The increase production of taurine at the expense of SO_4 excretion could be one way of reducing the acid load ("H" that would be produced if cysteine was catabolised to SO_4) since H^+ is not generated when taurine is synthesised from cysteine. To conclude rats can alleviate the acidosis caused by addition of sulphur amino acids to the diet by increasing the synthesis of glutamine at the expense of urea, and by increasing taurine production at the expense of SO_4.

Consideration of the relationship between GSH and urinary SO_4 during changes in dietary protein and sulphur amino acid intake and inflammation gives an insight into how the partitioning of sulphur amino acids between biosynthetic and catabolic pathways might change in response to alteration in dietary protein and sulphur amino acid intake, and inflammation. Despite the fact that the pair-fed animals were fed the same amount of food given to the corresponding LPS-treated animals, all dietary groups increased the ratio of total hepatic GSH to SO_4 excretion after exposure to the inflammatory response of LPS (Table 5.15). These results indicate that LPS treatment increased the flow of sulphur amino acids, coming from diet and from endogenous sources, towards GSH synthesis rather than to be catabolised to SO_4. In animals fed *ad libitum* and in LPS-treated animals, addition of methionine to the low-protein diets increased the ratio of total hepatic GSH to SO_4 excretion when compared with animals fed the same diets, but without methionine supplementation in the previous study (Table 4.16, chapter 4 & Table 5.15). The increase in the ratio, in the LPS-treated and in *ad libitum*-fed animals, could explain the dramatic increase in hepatic GSH concentration after addition of methionine to the low-protein diets. Furthermore, the increase in the ratio could also explain a possible increase in the catabolism of cysteine to taurine at the expense of

SO_4 when rats are fed a low-protein diet supplemented with sulphur amino acids, as mentioned earlier. However, in the pair-fed controls, addition of methionine to the low-protein diets reduced the ratio of hepatic GSH to SO_4 excretion. The reduction in the ratio after imposed reduction in food intake (low sulphur amino acids intake) may indicate that after food restriction, the flow of cysteine towards SO_4 is increased to a greater extent, at the expense of taurine and GSH synthesis. In animals fed the normal-protein diet, addition of methionine reduced the ratio in all treatment groups. This may suggest that most of the additional sulphur amino acids added to the normal-protein diet was catabolised to SO_4, and possibly taurine, rather than being used for GSH synthesis. This indication was supported by the following results, the average daily weight gain, and hepatic and lung protein content, which were similar between the NP group and the NP+M groups.

In the present study, it is clear that methionine supplementation has a major impact on tissue glutathione content in all treatment groups fed diets containing inadequate amounts of protein. There are a number of consequences, which arise from the response of GSH to diet and inflammation. These relate to the role of GSH as a reservoir of cysteine, as an antioxidant, and as immunomodulator.

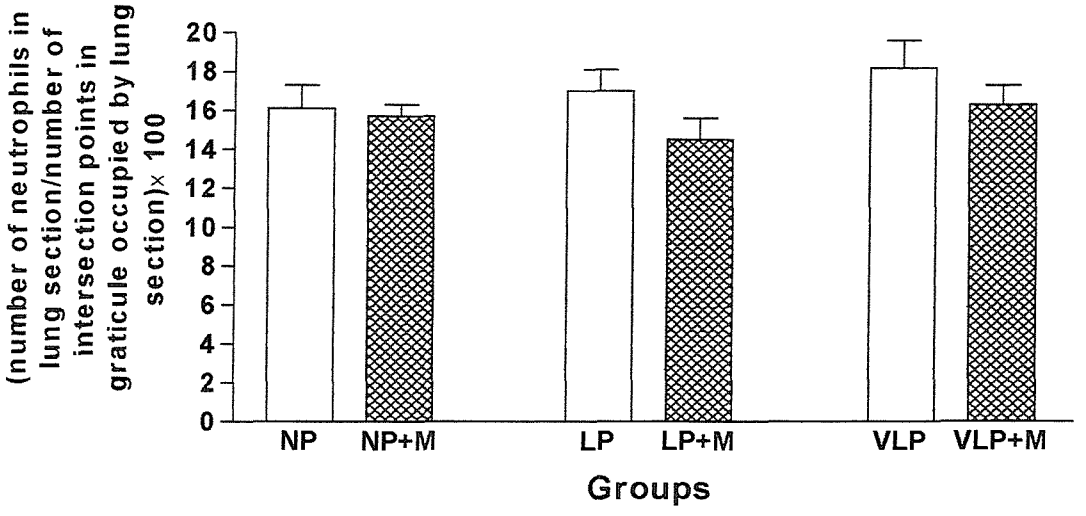
It could be suggested that addition of methionine to the very low-protein diet has applied a metabolic stress to the animals. Thus addition of methionine increased GSH concentration to prevent toxicity of cysteine. However, to increase GSH synthesis, glycine and glutamic acid are also needed. Consequently, other processes, which compete for glycine may be, disadvantaged (e.g. DNA, protein, and creatine synthesis). In addition, the conversion of homocysteine to cystathionine, in the first step of the transulphuration pathway needs serine to provide the carbon skeleton of cysteine. However, in the present study, comparing the relative amount of liver protein/kg body weight, it can be seen that liver protein, in proportion with body weight, was increased by addition of methionine, thus no impairment of protein synthesis is suggested. In addition, methionine supplementation may actually increase the relative amount of protein synthesis in the presence and absence of

inflammation. Thus, protein synthesis is not constrained by any potentially reduced availability of glycine or serine.

Glutathione may exert a major anti-inflammatory influence in the present study. In response to an inflammatory stimulus, free radicals (e.g. superoxide) and other oxidant molecules are produced by the immune system. Oxidant molecules, such as superoxide radical, hydrogen peroxide, hydrochlorous acid can be produced when phagocytic cells are activated (Droge *et al.* 1994; Halliwell *et al.* 1988). Although these molecules help to kill invading pathological organisms they carry the risk of destroying the host tissue by producing potentially toxic substances such as lipid peroxides, and by enhancing the production of cytokines indirectly by activating the nuclear transcription factor κB (NFκB). However, the presence of adequate amount of GSH will protect the body from these outcomes. In the present study, glutathione may be acting as a suppresser of NF-kB activation during inflammation, thereby decreasing the production of IL-8 (the neutrophil chemoattractent agent). This could explain the reduction (statistically not significant) in the number of neutrophils in lung of the LPS-treated animals fed the low-protein diets supplemented with methionine compared with animals fed the same diets, but without methionine supplementation (Figure 5-10). The number of neutrophils in lung in LPS-treated animals fed the LP and the VLP diets was 18% and 16% higher than animals fed diets with same protein content, but supplemented with methionine. Furthermore, the number of neutrophils in lung increased significantly in LPS-treated animals fed the low-protein diets (the LP and VLP diets) compared with the corresponding *ad libitum* and pair-fed animals, while in LPS-treated animals fed the low-protein diets supplemented with methionine (the LP+M and VLP+M diets) this effect was absent (see Table 4.21, chapter 4 & Table 5.20, chapter 5). The data presented on lung neutrophil number may thus be explainable in terms of the anti-inflammatory effect of raised and maintained tissue GSH concentration and the pro-inflammatory effect of low concentrations of the antioxidant. Neutrophils are attached to the site of tissue damage by the release of chemotactic agents. IL-8 is one of the major chemoattractent agents. Binding of IL-8 to IL-8 receptors on neutrophils

allows activation of the cell surface molecules, the integrin molecules. As a result of this activation, firm adhesion of neutrophils to the endothelium cells occurs. Consequently, neutrophils would be able to migrate between the endothelial cells to the site of inflammation. Moreover, IL-8 also activates neutrophils, causing a respiratory burst (Male *et al.* 1996). Thus the presence of large number of neutrophils at the site of inflammation could cause further damage to the tissues, due to release of free radicals, cytokines, and proteolysis enzymes. De Froge *et al.* (1992) have shown that addition of antioxidant-sulphur containing compounds, such as dimethyl sulfoxide (DMSO), dimethyl thiourea, and thiourea, significantly reduced the production of IL-8 by human blood in response to LPS stimulation. Furthermore, De Froge and his colleagues (1992) have also found that blood exposed to an oxidative stress of H_2O_2 increases production of IL-8, and that the addition of DMSO has opposite effect. Pena *et al.* (1999) found that GSH concentration in whole blood was low in cirrhotic patients. In vivo treatment with GSH prodrug, Oxothiazalidine-4-carboxylate that is converted to cysteine within the cell by the enzyme 5-oxoprolinase, significantly increase GSH concentration. The increase in GSH concentration was accompanied by a decrease in monocyte production of IL-8, IL-1, and TNF.

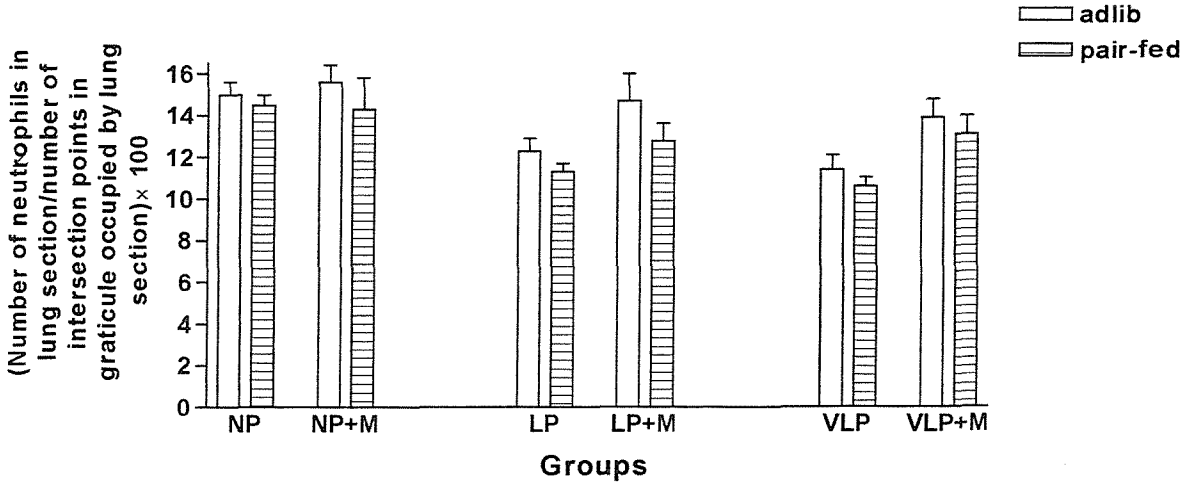
Figure 5-10 Number of neutrophils in lung in LPS-treated animals fed graded levels of casein with and without methionine supplementation



- Results are means ± SE. N=4 per treatment group.

Finally, in the present study, glutathione may be acting as an immunomodulator. It is well established that an adequate amount of glutathione is important for T-cells to function properly (Gmunder *et al.* 1990; Robinson *et al.* 1993; Fidelus & Tsan, 1986). For example, Gmunder *et al.* (1990) have shown that lymphocytes with high GSH concentration had increased DNA synthesis compared with lymphocytes with low GSH concentration. Robinson *et al.* (1993) showed that there was a significant reduction in the lymphocyte proliferation in both spleen and mesenteric lymph nodes in GSH depleted rats compared with the control rats, again indicating the importance of GSH in optimising T-cells and macrophage immune function. Thus, the role of glutathione, and cysteine in optimising T-cell function, and enhance lymphocyte proliferation could explain the increase (statistically not significant) in the number of neutrophils in lung in the *ad libitum* and pair-fed animals, given the low-protein diets supplemented methionine, compared with animals fed the low-protein diets without added methionine (Figure 5-11). The number of neutrophils in lung of animals fed *ad libitum* the LP and the VLP diets was 16% and 18% lower than *ad libitum*-fed animals given the LP+M and the VLP+M diets, respectively. For the pair-fed animals, the number of neutrophils was 12% and 19% lower than the pair-fed controls given the LP+M and the VLP+M diets, respectively.

Figure 5-11 Number of neutrophils in lung in *ad libitum* and pair-fed animals given graded levels of casein with and without methionine supplementation



- Results are means ± SE. N=4 per treatment group.

To conclude, the results of the present study showed that GSH concentration in liver, lung, spleen and thymus is sensitive to dietary sulphur amino acid intake. Of the four tissues, liver GSH is the most sensitive to dietary intake. The work described in the previous chapter (chapter 4) showed that when an inflammatory stress is applied to rats, not only is the ability to increase hepatic GSH impaired when sulphur amino acid intake is low, but that decreases in concentrations occur in lung, spleen and thymus. Dietary methionine supplementation facilitates increases in hepatic GSH concentrations and prevents the decreases in the other three tissues. Moreover, dietary methionine supplementation improves the growth of animals fed the low-protein diets. Addition of methionine to the animals fed the low-protein dietary groups significantly increased hepatic protein content and enhanced the production of the acute phase protein, α-1 acid glycoprotein, to a greater extent than animals fed the same protein content, but without methionine supplementation. Furthermore, addition of methionine to the low-protein dietary groups modulates the number of neutrophils in lung.

Therefore, the data presented in this chapter strongly suggest that even under poor protein nutrition status, e.g. malnutrition, in which the risk of a poor response to infection is high, the body's immunity may be improved by supplementing low-protein diets with methionine, and highlights the importance of sulphur amino acids during inflammation. In particular, the increase in GSH concentration in endotoxin-treated animals fed the low-protein diets supplemented with methionine may play a part in improving antioxidant defences, regulating cytokine production, and controlling inflammation.

CHAPTER SIX

6. The effect of graded levels of dietary casein but with identical amounts of sulphur amino acids and tryptophan on growth and inflammatory responsiveness in rats

6.1 Introduction

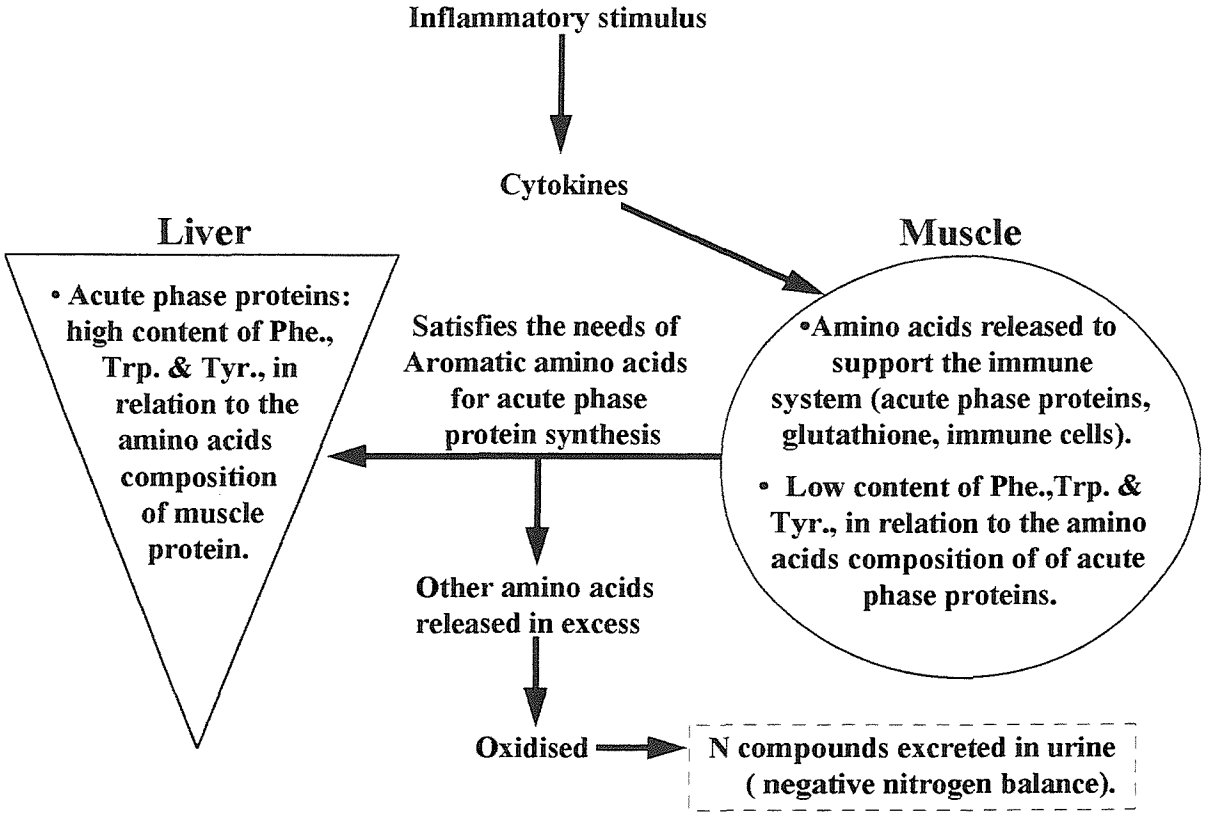
Chapter 5 shows the strong effect of supplementing the low-protein diets with methionine in enhancing growth, glutathione concentration in liver, lung, spleen and thymus, hepatic total protein and relative amount of protein in the liver in unstressed animals fed *ad libitum*. The supplement also exerted major enhancement of many of these parameters in animals fed the low-protein diets and undergoing an acute inflammatory response to LPS. The general conclusion from the study was that dietary sulphur amino acid sufficiency was important in both growth and in the acute inflammatory response.

However, Reeds *et al.* (1994) have calculated that the profile of amino acids released from skeletal muscle, the amino acid efflux, during the acute phase response is relatively deficient, in the aromatic amino acids (phenylalanine, tyrosine and tryptophan), in relation to the amino acid composition of the acute phase proteins. These authors suggest that the amino acid composition of the skeletal muscle protein does not match the amino acid requirement for synthesising acute phase proteins during the acute inflammatory response. As a result of this mismatch, when muscle protein is catabolised in amount that is sufficient for the acute phase protein response, some amino acids will be released in quantities that are surplus to these requirements. The surplus of amino acids are catabolised, contributing to the negative nitrogen balance which is characteristic of the infected, traumatised individual (Figure 6-1).

In the present study, the aromatic amino acid, tryptophan, which was suggested by Reeds *et al.* (1994) to be one of the most limiting amino acid for an

inflammatory response, was added to the low-protein diets supplemented with methionine, so that all dietary groups contain equal amounts of sulphur amino acids and of tryptophan (see Table 6.1). Inclusion of tryptophan in the low-protein diets, in addition to methionine supplementation, would not be expected to improve growth to a greater extent than methionine supplementation alone. However, additional enhancement of components of the inflammatory response should take place if tryptophan is limiting. Furthermore, in the present experiment the addition of tryptophan to the low-protein diets may reduced the demands made by inflammatory process upon the host's protein metabolism, thereby improving nitrogen balance if Reeds *et al.*'s (1994) hypothesis is correct.

Figure 6-1 The Reeds hypothesis



Keys; Phe.: Phenylalanine, Trp.: Tryptophan, Tyr.: Tyrosine.

6.2 Tryptophan content of the various diets

Diets were prepared by mixing dry ingredients in a mixer, this was followed by addition of maize oil. To these diets, water was added to make the diets into small biscuits that were dried in oven at 80 °C for up to 48-72 hours.

In order to achieve the aim of this study and highlight the importance of tryptophan sufficiency, rats were divided into three different groups. The amount of methionine and tryptophan added to all low-protein diets used in this study was equivalent to the amount of sulphur amino acids and tryptophan present in the NP+M group. Thus, any influence of sulphur amino acids and tryptophan adequacy in the presence of varying quantities of other amino acids would be apparent (see Table 6.1), by comparison with responses presented in chapters 4 & 5.

1) The first group, received 180 g protein supplemented with 3 g L-methionine/kg diet. This diet contains 65 mmol sulphur amino acids and 10.7 mmol tryptophan/kg diet, and this diet was used as it a customary diet for young growing rats. This group is called the normal-protein + L-methionine (NP+M) group.

2) The second group received 80 g protein supplemented with 6.70 g L-methionine and 1.20 g L-tryptophan/kg diet. This diet contains 65 mmol sulphur amino acids and 10.7 mmol tryptophan/kg diet. This group is called the low-protein + L-methionine + L-tryptophan (LP+M+T) group.

3) The third group received 60 g protein supplemented with 7.45 g L-methionine and 1.45 g L-tryptophan/kg diet. This diet contains 65 mmol sulphur amino acids and 10.7 mmol L-tryptophan/kg diet. This group is called the very low-protein + L-methionine + L-tryptophan (VLP+M+T) group.

6.3 Experimental protocol

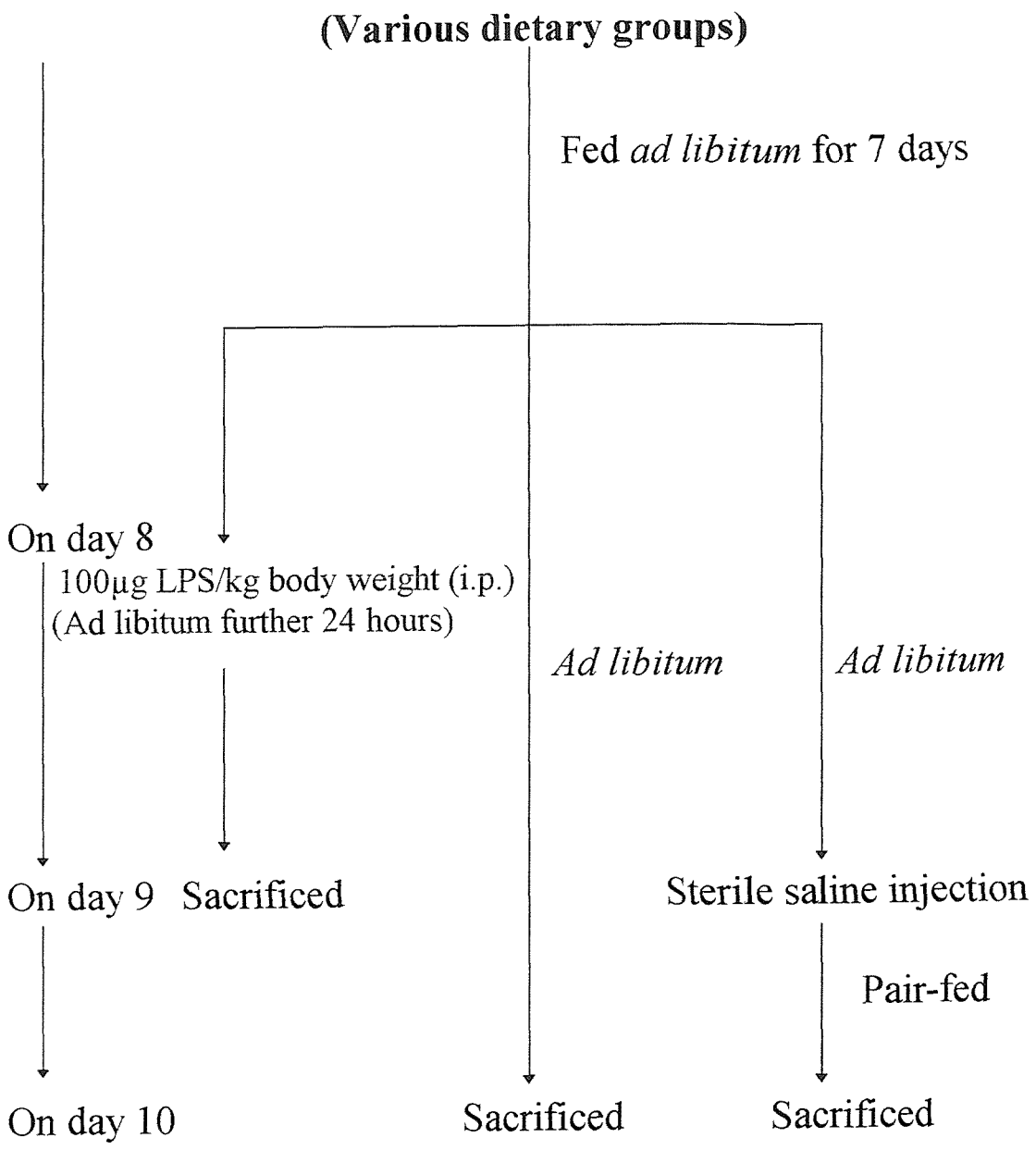
Male weanling wistar rats from Southampton University Medical School Colony weighing 50-70 g were housed individually in plastic metabolic cages and were maintained at 22 ± 1°C with a 12-12 hour light-dark cycle (Lights on at 7:30 AM).

Rats were divided into three different dietary groups (n=12 for the NP+M group, and n=18 for the LP+M+T and the VLP+M+T groups) and fed *ad libitum* the semi-synthetic diets for 7 days. On day 8, animals from each group were subdivided into three treatment groups, and fed, injected and biopsied as described in chapter 4, section 4.3 (see Figure 6-2).

Table 6.1 Diets composition (g/kg)

Component	180 g protein/kg	80 g protein/kg	60 g protein/kg
Casein	204	91	68
L-methionine	3	6.70	7.45
L-tryptophan	0	1.20	1.45
Cellulose	100	100	100
Sucrose	319	373	384
Maize starch	319	373	384
Maize oil	30	30	30
Vitamin mix	5	5	5
Mineral mix	20	20	20

Figure 6-2 Schematic representation of the experimental protocol



6.4 Results

6.4.1 Growth, food intake, sulphur amino acid intake, and organ weights

Average daily weight gain, food intake, sulphur amino acid intake, and food, energy and protein efficiencies before starting treatments

There was a significant reduction in the average daily weight gain as dietary protein content was decreased (Table 6.2). Addition of tryptophan did not further improve the growth performance of rats fed the low-protein diets supplemented with methionine. The average daily weight gain of rats fed the LP+M+T and the VLP+M+T diets was 5.0 g and 3.4g/day respectively, while in the previous study (chapter 5), the average daily weight gain of rats fed the LP+M and VLP+M diets was 4.9 g and 3.6 g/day respectively.

No significant difference was found in the food intake between the groups (Table 6.2). Thus, as in the previous experiment, no difference in sulphur amino acids intake was found between the groups, before starting treatments (Table 6.2). The food efficiency decreased as dietary protein content in the diet was decreased (Table 6.2). Addition of tryptophan to the low-protein diets supplemented with methionine did not improve the food efficiency further (Table 5.2, chapter 5 & Table 6.2). The food efficiency (g weight gain/g food eaten) in the LP+M+T and the VLP+M+T groups was 0.36 and 0.27 respectively, and in the previous experiment, it was 0.37 and 0.27 respectively for the LP+M and the VLP+M groups.

The energy efficiency (mg weight gain/kJ eaten) decreased as dietary protein content in the diet was decreased (Table 6.2). Addition of tryptophan to the low-protein diets supplemented with methionine did not improve the energy efficiency further when compared to values from animals fed the LP+M and VLP+M diets in the previous experiment (Table 5.2, chapter 5 & Table 6.2).

The efficiency with which dietary protein was used for growth, protein efficiency (g weight gain/g protein eaten), in animals fed the LP+M+T and

VLP+M+T diets was significantly higher than in animals fed the NP+M diet (Table 6.2). Moreover, addition of tryptophan to the low-protein diets supplemented with methionine did not improve the protein efficiency further. The protein efficiency in animals fed the LP+M+T and VLP+M+T diets and in the corresponding animals fed the LP+M and VLP+M diets in the previous experiment was similar (see Table 5.2, chapter 5 & Table 6.2).

Table 6.2 Average daily weight gain, food intake, sulphur amino acid intake, and food, protein and energy efficiency of rats fed diets of graded casein content but with identical amounts of sulphur amino acids and tryptophan, before starting treatments

Dietary groups g protein/kg diet	NP+M 180+M	LP+M+T 80+M+T	VLP+M+T 60+M+T	one-way ANOVA p-value	 F ratio
weight gain (g)	6.4 ± 0.22[a]	5.0 ± 0.14[b]	3.4 ± 0.24[c]	p=0.000	F=49
Food intake (g)	13.4 ± 0.35[a]	13.66 ± 0.34[a]	12.6 ± 0.41[a]	p=0.108	F=2.34
sulphur amino acid intake (µmol)	871 ± 23[a]	888 ± 22[a]	820 ± 26[a]	p=0.108	F=2.34
weight gain/food intake (g/g)	0.47 ± 0.01[a]	0.36 ± 0.01[b]	0.27 ± 0.01[c]	p=0.000	F=73.56
weight gain/energy intake (mg/kJ)	26.0 ± 0.70[a]	20.2 ± 0.48[b]	14.8 ± 0.76[c]	p=0.000	F=73.56
weight gain/protein intake (g/g)	2.6 ± 0.06[a]	4.5 ± 0.11[b]	4.4 ± 0.23[b]	p=0.000	F=34.38

-Results are presented as mean ± standard error of the mean, n=12 for the NP+M group, and n=18 for the LP+M+T and the VLP+M+T groups. Means within each row having different letter superscripts following the number differ significantly.

The effect of LPS on food intake and sulphur amino acid intake after starting treatments

In animals fed *ad libitum*, no significant difference in food intake (and sulphur amino acid intake) was found between the groups. In the LPS-treated rats and pair-fed controls, no significant difference in food intake (and sulphur amino

acid intake) was found between the groups, However, the mean values was higher in the VLP+M+T compared with other groups (Table 6.3).

A significant reduction in food (and sulphur amino acid intake) was found in the LPS treated and pair-fed animals compared with the corresponding *ad libitum*-fed animals in the LP+M+T and the VLP+M+T groups. In the NP+M group, the mean values was lower after LPS treatment and pair-feeding, but due to large inter-group variability, the decrease in food intake (and sulphur amino acid intake) was not statistically significant.

Table 6.3 The effect of LPS on food intake (g/day) and sulphur amino acid intake (μmol/day) of rats fed diets of graded casein content but with identical amounts of sulphur amino acids and tryptophan, after starting treatments

Dietary groups g protein/kg diet	NP+M 180+M	LP+M+T 80+M+T	VLP+M+T 60+M+T	two-way ANOVA p-value	 F-ratio
Food intake					
ad libitum	15.1 ± 0.6 [a]	15.1 ± 1.1 [a]	14.4 ± 0.6 [a]	D=0.148	F=2.01
pair-fed	7.2 ± 2.5 [a]	5.6 ± 1.2 [a]*	9.4 ± 0.3 [a]*	T=0.000	F=24.94
LPS	6.9 ± 2.5 [a]	5.4 ± 2.1 [a]*	9.1 ± 1.2 [a]*	I=0.488	F=0.87
Sulphur amino acid intake					
ad libitum	980 ± 40 [a]	986 ± 73 [a]	940 ± 38 [a]	D=0.148	F=2.01
pair-fed	469 ± 164 [a]	366 ± 77 [a]*	615 ± 17 [a]*	T=0.000	F=24.94
LPS	449 ± 193 [a]	349 ± 140 [a]*	596 ± 82 [a]*	I=0.488	F=0.87

-Results are presented as mean ± standard error of the mean, n=4 per treatment group for animals fed the NP+M diet, and n=6 per treatment group for animals fed the LP+M+T and the VLP+M+T diets. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

Final body weight

The results of the final body weight can be seen in Table 6.4. In animals fed *ad libitum*, there was a trend for a reduction in the final body weight as dietary protein intake was decreased. This difference was statistically significant when

comparing the NP+M group with the VLP+M+T group. In the LPS treated and pair-fed animals, the mean values of the final weight was decreased as dietary protein content was decreased. This difference was statistically significant in the pair-fed animals when comparing the NP+M group with the VLP+M+T group. Due to treatments, the mean values of the final body weight decreased in the LPS treated and pair-fed animals compared with the animals fed *ad libitum*, but inter-group variability was such that this difference was only significant when comparing the LPS-treated animals with the *ad libitum*-fed animals given the LP+M+T and the VLP+M+T diets.

Table 6.4 Final body weight of rats fed diets of graded casein content but with identical amounts of sulphur amino acids and tryptophan

Dietary groups g protein/kg diet	NP+M 180+M	LP+M+T 80+M+T	VLP+M+T 60+M+T	two-way ANOVA p-value	 F-ratio
Final body weight (g)					
ad libitum	120.0 ± 3.1 [a]	106.9 ± 5.0 [a]	90.96 ± 2.7 [b]	D=0.000	F=17.50
pair-fed	104.5 ± 6.2 [a]	93.8 ± 4.4 [ab]	82.0 ± 3.9 [b]	T=0.001	F=9.31
LPS	100.8 ± 7.7 [a]	88.0 ± 6.2 [a]*	80.0 ± 3.3 [a]*	I=0.888	F=0.28

-Results are presented as mean ± standard error of the mean, n=4 per treatment group for animals fed the NP+M diet, and n=6 per treatment group for animals fed the LP+M+T and the VLP+M+T diets. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

Organ weights

a) Total liver weight

No significant difference in total liver weight was found between the groups due to diet.

Due to treatments, in all dietary groups, the pair-fed animals had a lower total liver weight compared with the corresponding animals fed *ad libitum*. In the LP+M+T and VLP+M+T groups, LPS treated-animals had a lower total liver weight compared with the corresponding animals fed *ad libitum*. The total liver weight of

LPS-treated animals fed the VLP+M+T diet was also higher than the corresponding pair-fed controls (Table 6.5).

b) Relative liver weight

In animals fed *ad libitum* and in LPS-treated animals, there was a significant increase in the relative liver weight (g/kg body weight) in the LP+M+T and VLP+M+T groups compared with the NP+M group. In the pair-fed controls, there was a trend for an increase in the relative liver weight, but this increase was not statistically significant (Table 6.5).

In all dietary groups, the LPS-treated animals had higher mean values than the corresponding pair-fed controls. The difference was statistically significant in the LP+M+T and VLP+M+T groups. The LPS-treated rats and pair-fed controls in the LP+M+T and VLP+M+T groups had a lower relative weight compared with the corresponding animals fed *ad libitum*. The addition of tryptophan to the low-protein dietary groups supplemented with methionine (The LP+M+T and VLP+M+T groups) in animals fed *ad libitum*, LPS-treated rats, and in pair-fed controls had no effect on the relative liver weight compared with animals fed the same diets, but without tryptophan supplementation in the previous study (see Table 5.5; chapter 5 & Table 6.5).

c) Total lung weight

In the animals fed *ad libitum*, LPS-treated rats and pair-fed groups, there was a trend for a reduction in the total lung weight as dietary protein content was decreased. This reduction was statistically significant in the *ad libitum* and in LPS-treated animals when comparing the NP+M group with the VLP+M+T group, and in the pair-fed animals, when comparing the NP+M and the LP+M+T groups with the VLP+M+T group (Table 6.5).

Total lung weight in the NP+M group was significantly lower in the LPS-treated animals compared with animals fed *ad libitum*.

d) Relative lung weight

As in the previous experiment, neither diet nor treatments caused a significant difference in the relative lung weight (g/kg body weight) between the groups (see Table 5.5; chapter 5 & Table 6.5).

e) Total spleen weight

In animals fed *ad libitum*, there was a significant reduction in the total spleen weight as dietary protein content was decreased. In LPS treated and in pair-fed animals, there was a trend for a reduction in total spleen weight as dietary protein content was decreased (Table 6.5). This difference was statistically significant when comparing the NP+M and LP+M+T groups with the VLP+M+T group in pair-fed animals, and when comparing the NP+M group with the VLP+M+T group in LPS-treated animals. In the NP+M group, Pair-fed animals had lower spleen weight compared with animals fed *ad libitum*.

f) Relative spleen weight

In animals fed *ad libitum*, the relative spleen weight (g/kg body weight) decreased significantly as dietary protein content was decreased. In pair-fed and in LPS-treated animals, relative spleen weight was significantly lower in the VLP+M+T group compared to the NP+M group. In the VLP+M+T group, LPS-treated animals had significantly higher relative spleen weight compared with *ad libitum* and pair-fed animals. In the LP+M+T group, LPS-treated animals had a significantly higher relative spleen weight compared with animals fed *ad libitum* (Table 6.5).

The addition of tryptophan to the low-protein dietary groups supplemented with methionine (the LP+M+T and VLP+M+T groups) had no effect on the relative spleen weight compared with the low-protein dietary groups supplemented with methionine (the LP+M and VLP+M groups) in the previous experiment (see Table 5.5; chapter 5 & Table 6.5).

g) Total thymus weight

The total thymus weight was not severely affected by the reduction in dietary protein content, unlike the total spleen weight (Table 6.5).

In animals fed *ad libitum*, there was a trend for a reduction in total thymus weight as dietary protein content was decreased. This difference was statistically significant when comparing the NP+M group with the VLP+M+T group. In the LPS-treated and pair-fed animals, the mean values decreased as dietary protein content was decreased, but this difference was not statistically significant. Table 6.5 shows that LPS-treated animals fed the VLP+M+T diet had lower thymus weight compared with their corresponding animals fed *ad libitum*, and that pair-fed animals fed the LP+M+T diet had lower thymus weight compared with their corresponding animals fed *ad libitum*.

h) Relative thymus weight

As in the previous study, neither diet nor treatments caused a significant alteration in the relative thymus weight (g/kg body weight) (see Table 6.5).

Addition of tryptophan to the low-protein diets supplemented with methionine had no effect on the relative thymus weight.

Table 6.5 Organ weights of rats fed diets of graded casein content but with identical amounts of sulphur amino acids and tryptophan, after starting treatments

Dietary groups g protein/kg diet	NP+M 180+M	LP+M+T 80+M+T	VLP+M+T 60+M+T	two-way ANOVA p-value	F ratio
Total liver weight (g)					
ad libitum	5.80 ± 0.25 ^{a}	6.73 ± 0.27 ^{a}	6.17 ± 0.17 ^{a}	D=0.101	F=2.43
pair-fed	4.60 ± 0.19 ^{a}*	4.40 ± 0.16 ^{a}*	4.14 ± 0.20 ^{a}*	T=0.000	F=44.29
LPS	5.00 ± 0.22 ^{a}	5.13 ± 0.39 ^{a}*	4.74 ± 0.15 ^{a}♠*	I=0.247	F=1.41
Relative liver weight (g/kg body weight)					
ad libitum	48.3 ± 1.9 ^{a}	63.1 ± 1.5 ^{b}	67.9 ± 1.8 ^{b}	D=0.000	F=24.82
pair-fed	44.3 ± 1.9 ^{a}	46.7 ± 1.6 ^{a}*	50.9 ± 2.9 ^{a}*	T=0.000	F=31.21
LPS	50.8 ± 1.9 ^{a}	58.2 ± 0.9 ^{b}♠*	59.1 ± 2.0 ^{b}♠*	I=0.013	F=3.63
Total lung weight (mg)					
ad libitum	966 ± 22 ^{a}	879 ± 50 ^{ab}	767 ± 34 ^{b}	D=0.000	F=20.15
pair-fed	902 ± 45 ^{a}	787 ± 16 ^{a}	678 ± 34 ^{b}	T=0.001	F=8.86
LPS	828 ± 32 ^{a}*	748 ± 25 ^{ab}	659 ± 47 ^{b}	I=0.963	F=0.15
Relative lung weight (g/kg body weight)					
ad libitum	8.07 ± 0.32 ^{a}	8.23 ± 0.34 ^{a}	8.47 ± 0.45 ^{a}	D=0.928	F=0.08
pair-fed	8.68 ± 0.40 ^{a}	8.48 ± 0.25 ^{a}	8.31 ± 0.39 ^{a}	T=0.800	F=0.22
LPS	8.30 ± 0.45 ^{a}	8.65 ± 0.53 ^{a}	8.23 ± 0.44 ^{a}	I=0.869	F=0.31
Total spleen weight (mg)					
ad libitum	620 ± 25 ^{a}	427 ± 22 ^{b}	300 ± 22 ^{c}	D=0.000	F=39.44
pair-fed	478 ± 38 ^{a}*	386 ± 22 ^{a}	282 ± 14 ^{b}	T=0.016	F=4.62
LPS	515 ± 39 ^{a}	476 ± 50 ^{ab}	350 ± 25 ^{b}	I=0.107	F=2.05
Relative spleen weight (g/kg body weight)					
ad libitum	5.16 ± 0.19 ^{a}	3.99 ± 0.06 ^{b}	3.28 ± 0.17 ^{c}	D=0.000	F=27.47
pair-fed	4.59 ± 0.31 ^{a}	4.15 ± 0.30 ^{ab}	3.45 ± 0.14 ^{b}	T=0.000	F=10.79
LPS	5.12 ± 0.11 ^{a}	4.84 ± 0.15 ^{ab}*	4.38 ± 0.26 ^{b}♠*	I=0.096	F=2.13
Total thymus weight (mg)					
ad libitum	756 ± 74 ^{a}	619 ± 44 ^{ab}	510 ± 20 ^{b}	D=0.000	F=12.61
pair-fed	576 ± 59 ^{a}	490 ± 22 ^{a} *	472 ± 32 ^{a}	T=0.001	F=8.50
LPS	589 ± 59 ^{a}	511 ± 44 ^{a}	419 ± 22 ^{a}*	I=0.514	F=0.83
Relative thymus weight (g/kg body weight)					
ad libitum	6.44 ± 0.80 ^{a}	5.83 ± 0.41 ^{a}	5.62 ± 0.18 ^{a}	D=0.409	F=0.92
pair-fed	5.48 ± 0.28 ^{a}	5.24 ± 0.14 ^{a}	5.75 ± 0.26 ^{a}	T=0.224	F=1.56
LPS	5.81 ± 0.16 ^{a}	5.81 ± 0.36 ^{a}	5.24 ± 0.19 ^{a}	I=0.365	F=1.11

-Results are presented as mean ± standard error of the mean, n=4 per treatment group for animals fed the NP+M diet, and n=6 per treatment group for animals fed the LP+M+T and the VLP+M+T diets. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

6.4.2 Glutathione concentration, and glutathione peroxidase and reductase activities

Glutathione concentration

a) Hepatic glutathione concentration

Hepatic glutathione (GSH) concentration was influenced by dietary protein content, food intake and LPS treatment (Table 6.6 & Figure 6-3). In the *ad libitum* and pair-fed animals, hepatic GSH concentration was significantly higher in the VLP+M+T group compared with the NP+M and the LP+M+T groups. In the LPS-treated animals, no significant difference in GSH concentration was found between the groups. In all the groups, rats receiving LPS had a higher GSH concentration compared with the corresponding pair-fed controls.

Comparison with data presented in the previous experiment, it can be seen that addition of tryptophan to the low-protein dietary groups supplemented with methionine slightly increased liver GSH in *ad libitum*-fed animals (from 5.66 μmol/g liver in the LP+M group to 6.44 μmol/g liver in the LP+M+T group, and from 7.86 μmol/g liver in the VLP+M group to 8.87 μmol/g liver in the VLP+M+T group). However, the difference was not statistically significant. In rats given LPS, addition of tryptophan to the low-protein dietary groups supplemented with methionine slightly increased hepatic GSH concentration in the animals fed the very-low protein diet (from 7.52 μmol/g liver in the VLP+M group to 8.21μmol/g liver in the VLP+M+T group). However, the difference was not statistically significant (see Table 5.6, chapter 5 & Table 6.6).

b) Lung glutathione concentration

No significant difference in lung GSH concentration was found due to diet as a factor.

Due to treatments, there was a significant increase in lung GSH concentration in all the groups after LPS injection when compared with the pair-fed controls (Table 6.6 & Figure 6-3).

Comparison the data with that from the previous chapter, the addition of tryptophan to the low-protein diets supplemented with methionine did not modify this effect (see Table 5.6, chapter 5 & Table 6.6).

c) Spleen glutathione concentration

LPS treated and *ad libitum*-fed animals given the VLP+M+T diet had lower spleen GSH concentration compared with animals given the NP+M diet. In the pair-fed groups, animals fed the VLP+M+T diet tends to have lower GSH concentration compared to other groups, but this difference was not statistically significant (Table 6.6 & Figure 6-3).

Comparison with work reported in the previous chapter, the addition of tryptophan to the low-protein diets supplemented with methionine did not modify this effect (see Table 5.6, chapter 5 & Table 6.6).

d) Thymus glutathione concentration

In all dietary groups, there was a reduction in thymus GSH concentration as dietary protein content was decreased. This difference was statistically significant in the LPS-treated animals when comparing the NP+M group with the VLP+M+T group (Table 6.6 & Figure 6-3).

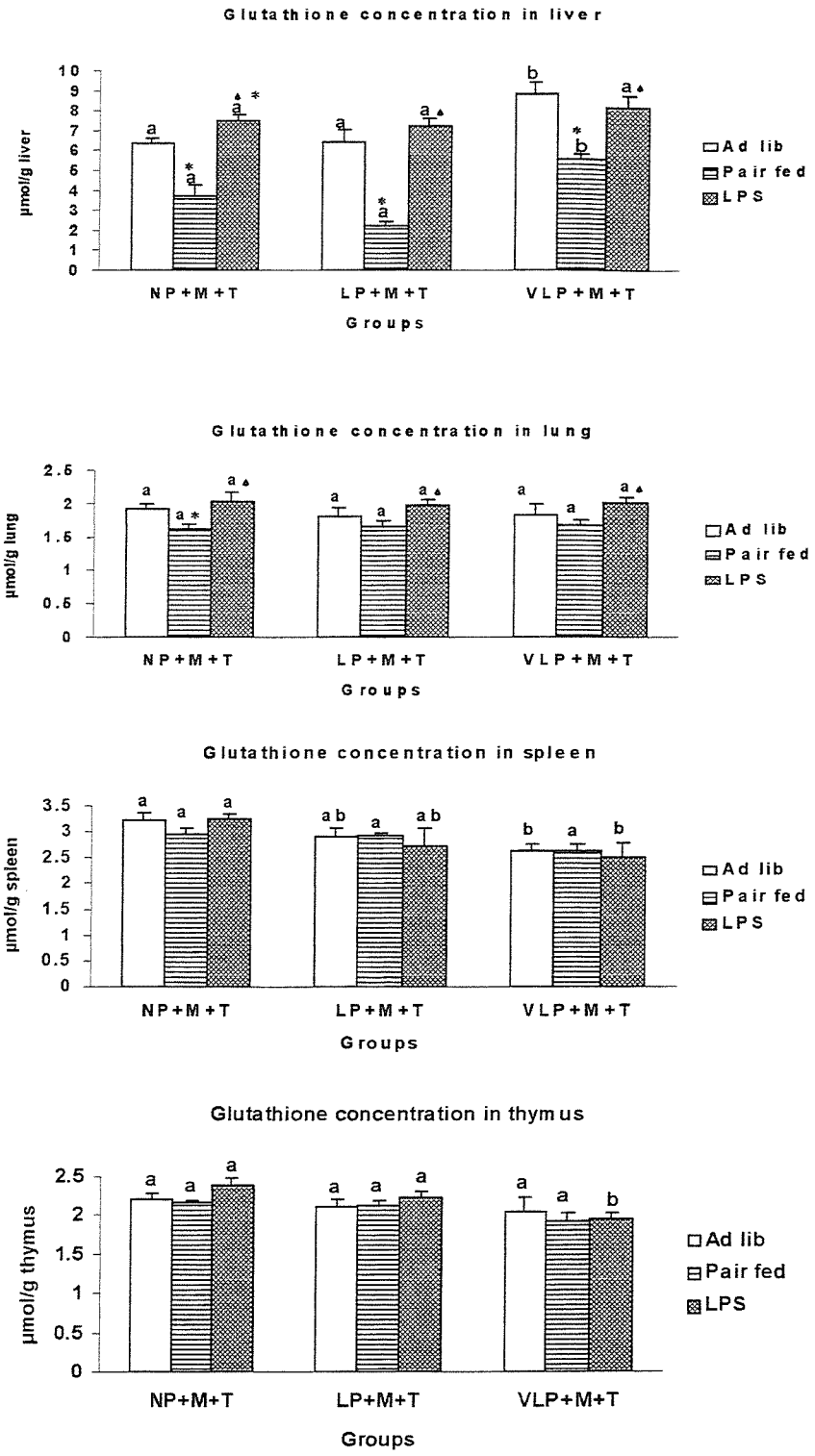
Addition of tryptophan to the low-protein diets supplemented with methionine exerted no modulatory influence (see Table 5.6, chapter 5 & Table 6.6).

Table 6.6 Glutathione concentration in liver, lung, spleen, and thymus of rats fed diets of graded casein content but with identical amounts of sulphur amino acids and tryptophan, after starting treatments (µmol/g tissue)

Dietary groups g protein/kg diet	NP+M 180+M	LP+M+T 80+M+T	VLP+M+T 60+M+T	two-way ANOVA p-value	 F ratio
Liver					
ad libitum	6.40 ± 0.21^{a}	6.44 ± 0.63^{a}	8.87 ± 0.57^{b}	D=0.000	F=20.66
pair-fed	3.75 ± 0.54^{a}*	2.24 ± 0.24^{a}*	5.63 ± 0.22^{b}*	T=0.000	F=56.30
LPS	7.52 ± 0.32^{a} ♠*	7.25 ± 0.40^{a}♠	8.21 ± 0.60^{a}♠	I=0.068	F=2.38
Lung					
ad libitum	1.93 ± 0.06^{a}	1.82 ± 0.13^{a}	1.84 ± 0.15^{a}	D=0.871	F=0.14
pair-fed	1.62 ± 0.07^{a}*	1.66 ± 0.09^{a}	1.67 ± 0.09^{a}	T=0.001	F=8.29
LPS	2.04 ± 0.13^{a}♠	1.97 ± 0.09^{a}♠	2.02 ± 0.08^{a}♠	I=0.968	F=0.14
Spleen					
ad libitum	3.23 ± 0.15^{a}	2.90 ± 0.18^{ab}	2.64 ± 0.12^{b}	D=0.013	F=4.85
pair-fed	2.95 ± 0.14^{a}	2.92 ± 0.06^{a}	2.64 ± 0.12^{a}	T=0.819	F=0.20
LPS	3.24 ± 0.11^{a}	2.73 ± 0.36^{ab}	2.51 ± 0.27^{b}	I=0.837	F=0.36
Thymus					
ad libitum	2.21 ± 0.08^{a}	2.10 ± 0.10^{a}	2.04 ± 0.18^{a}	D= 0.007	F=5.68
pair-fed	2.17 ± 0.02^{a}	2.12 ± 0.07^{a}	1.92 ± 0.10^{a}	T=0.373	F=1.01
LPS	2.39 ± 0.09^{a}	2.23 ± 0.08^{a}	1.94 ± 0.08^{b}	I=0.686	F=0.57

-Results are presented as mean ± standard error of the mean, n=4 per treatment group for animals fed the NP+M diet, and n=6 per treatment group for animals fed the LP+M+T and the VLP+M+T diets. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

Figure 6-3 Glutathione concentration in liver, lung, spleen, and thymus



-Results are means ± SE. N=4 per treatment group for animals fed the NP+M diet, and n=6 per treatment group for animals fed the LP+M and the VLP+M diets. Means within each treatment group having different letter superscripts differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

Glutathione peroxidase activity

a) Glutathione peroxidase activity in liver

In the LPS treated and in *ad libitum*-fed animals there was an increase in liver glutathione peroxidase (GPx) activity as dietary protein content was decreased, as in the previous experiment (see Tables 5.7, chapter 5 & Table 6.7).

Due to diet, hepatic GPx activity in the VLP+M+T group was significantly higher than in the NP+M group in both animals fed *ad libitum* and in LPS-treated animals. In the pair-fed animals, no difference in GPx activity was found between the groups.

Due to treatments, in the VLP+M+T group, GPx activity of the endotoxin-treated rats and animals fed *ad libitum* was significantly higher than the corresponding pair-fed controls.

Addition of tryptophan to the low-protein diets supplemented with methionine in the present study had no influence on liver GPx activity compared with previous study, in which animals fed the same diets but without additional tryptophan.

b) Glutathione peroxidase activity in lung, spleen, and thymus

Neither diet nor treatments caused an alteration on the activity of GPx in the above organs, indicating that GPx activity in these organs can be maintained during inflammation or consumption of a protein deficient diet (Table 6.7).

Addition of tryptophan to the low-protein diets supplemented with methionine in the present study had no influence on GPx activity in the above organs (see Tables 5.7, chapter 5 & Table 6.7).

Table 6.7 Glutathione peroxidase activity in liver, lung, spleen, and thymus of rats fed diets of graded casein content but with identical amounts of sulphur amino acids and tryptophan, after starting treatments (units/g protein)

Dietary groups g protein/kg diet	NP+M 180+M	LP+M+T 80+M+T	VLP+M+T 60+M+T	two-way ANOVA p-value	 F ratio
Liver					
ad libitum	29.5 ± 1.5 ^{a}	34.6 ± 1.3 ^{ab}	40.5 ± 0.3 ^{b}	D=0.011	F=4.5
pair-fed	27.8 ± 2.2 ^{a}	31.1 ± 1.9 ^{a}	30.1 ± 1.5 ^{a}*	T=0.008	F=5.3
LPS	31.4 ± 1.2^{a}	33.4 ± 2.9 ^{ab}	38.0 ± 0.6 ^{b}♠	I=0.063	F=1.9
Lung					
ad libitum	61.2 ± 0.6 ^{a}	59.7 ± 1.6 ^{a}	62.4 ± 0.8 ^{a}	D=0.152	F=2.2
pair-fed	58.8 ± 0.9 ^{a}	57.1 ± 0.9 ^{a}	61.5 ± 1.8 ^{a}	T=0.425	F=0.91
LPS	59.4 ± 1.7 ^{a}	58.8 ± 1.8 ^{a}	61.3 ± 1.2 ^{a}	I=0.495	F=0.96
Spleen					
ad libitum	62.6 ± 1.1 ^{a}	63.2 ± 2.2 ^{a}	64.8 ± 1.5 ^{a}	D=0.093	F=2.1
pair-fed	64.8 ± 1.6 ^{a}	62.5 ± 0.9 ^{a}	59.8 ± 2.2 ^{a}	T=0.25	F=1.55
LPS	63.4 ± 2.1 ^{a}	64.4 ± 1.5 ^{a}	63.7 ± 2.6 ^{a}	I=0.076	F=2.1
Thymus					
ad libitum	58.9 ± 0.9 ^{a}	59.9 ± 1.5 ^{a}	60.9 ± 1.7 ^{a}	D=0.41	F=0.91
pair-fed	59.7 ± 1.3 ^{a}	60.4 ± 2.8 ^{a}	61.6 ± 2.1 ^{a}	T=0.75	F= 0.19
LPS	63.6 ± 1.7 ^{a}	62.3 ± 2.1 ^{a}	65.9 ± 1.6 ^{a}	D=0.82	F=0.26

-Results are presented as mean ± standard error of the mean, n=4 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

Glutathione reductase activity

a) Glutathione reductase activity in liver

In animals fed *ad libitum* and in LPS-treated animals, feeding a diet severely deficient in protein caused a significant increase in glutathione reductase (GR) activity (Table 6.8).

In the animals fed *ad libitum*, GR activity was significantly higher in the VLP+M+T group compared with the NP+M and the LP+M+T groups. In the pair-fed animals, GR activity was similar between the groups. As in *ad libitum*-fed animals, GR activity in the LPS-treated animals was significantly higher in the VLP+M+T group compared with the NP+M and the LP+M+T groups.

Due to treatments, GR activity was similar between the groups. However, in the VLP+M+T group, there was a trend for an increase in GR activity occurred in the *ad libitum* and in LPS-treated animals compared with the corresponding pair-fed controls.

It can be seen, by comparing the results with those of the previous experiment (see Table 5.8, chapter 5 & Table 6.8), that addition of tryptophan had no effect on the activity of GR.

b) Glutathione reductase activity in lung, spleen, and thymus

Diet and inflammation had no effect on GR activity in these tissues. Comparison with data from the previous study, the addition of tryptophan had no effect on GR activity on the above organs (see Table 5.8, chapter 5 & Table 6.8).

Table 6.8 Glutathione reductase activity in liver, lung, spleen and thymus of rats fed diets of graded casein content but with identical amounts of sulphur amino acids and tryptophan, after starting treatments (units/mg protein)

Dietary groups g protein/kg diet	NP+M 180+M	LP+M+T 80+M+T	VLP+M+T 60+M+T	two-way ANOVA p-value F-ratio
Liver				
ad libitum	20.7 ± 1.2 [a]	20.4 ± 1.8 [a]	29.1 ± 2.2[b]	D=0.001 F=8.70
pair-fed	15.3 ± 1.4 [a]	18.0 ± 3.5 [a]	19.5 ± 1.8 [a]	T=0.171 F=1.90
LPS	17.1 ± 1.6[a]	19.3 ± 1.4 [a]	27.6 ± 1.8[b]	I=0.224 F=1.53
Lung				
ad libitum	22.5 ± 1.9 [a]	20.4 ± 0.9 [a]	24.3 ± 2.1 [a]	D=0.117 F=2.33
pair-fed	19.8 ± 2.6 [a]	24.5 ± 1.3 [a]	21.1 ± 1.1 [a]	T=0.391 F=0.97
LPS	18.7 ± 1.6 [a]	19.8 ± 0.9 [a]	23.7 ± 0.9 [a]	I=0.068 F=2.49
Spleen				
ad libitum	22.5 ± 2.0 [a]	23.45 ± 1.2 [a]	26.04 ± 2.0 [a]	D=0.060 F=3.16
pair-fed	21.2 ± 1.4 [a]	23.65 ± 2.2 [a]	25.2 ± 1.4 [a]	T=0.085 F=2.72
LPS	16.9 ± 1.5 [a]	21.22 ± 2.8 [a]	22.2 ± 4.5 [a]	I=0.957 F=0.16
Thymus				
ad libitum	22.5 ± 1.0 [a]	22.8 ± 1.7 [a]	23.9 ± 1.3 [a]	D=0.396 F=0.96
pair-fed	21.1 ± 1.2 [a]	21.8 ± 1.2 [a]	19.2 ± 2.4 [a]	T=0.174 F=1.87
LPS	20.1 ± 1.0 [a]	21.9 ± 0.4 [a]	22.1 ± 2.2 [a]	I=0.231 F=1.50

-Results are presented as mean ± standard error of the mean, n=4 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

6.4.3 Protein in liver and lung

a) Liver protein concentration

In the animals fed *ad libitum* and in LPS-treated animals, there was a significant reduction in protein concentration as dietary protein content was decreased, which was most severe when the protein concentration in the diet contained 60 g/kg diet, as in the previous study. In the pair-fed animals, there was a trend for a reduction in protein concentration as dietary protein content was decreased, but this reduction was not statistically significant (Table 6.9). In the NP+M group, LPS-treated animals and pair-fed animals had a higher protein concentration than in the *ad libitum*-fed animals.

Addition of tryptophan to the low-protein diets supplemented with methionine exerted no influence on the results (see Table 5.9, chapter 5 & Table 6.9).

b) Total liver protein

Dietary protein content, food intake and LPS treatment influenced the total liver protein content. In general, total liver protein among animals of various dietary and treatment groups varied due to the difference in the weights of livers (Table 6.9).

In the LPS treated and pair-fed animals, there was a trend for a reduction in the total liver protein as dietary protein content was decreased. This was statistically significant in the LPS and pair-fed animals when comparing the NP+M group with the VLP+M+T group. In animals fed *ad libitum*, it was lower in the VLP+M+T group than in the LP+M+T group.

Comparing the LPS-treated rats with pair-fed controls, it can be seen that in all the groups, the mean values was higher in the LPS-treated animals compared with the corresponding pair-fed controls. In the LP+M+T group, Pair-fed animals had a lower total liver protein than in *ad libitum*-fed animals. In the VLP+M+T group, it was lower in the LPS treated and pair-fed animals compared with animals fed *ad libitum*.

Addition of tryptophan to the low-protein diets supplemented with methionine exerted no influence on the total protein in liver (see Table 5.9, chapter 5 & Table 6.9).

c) Total liver protein/kg body weight

When the total liver protein is expressed relative to body weight it can be seen that, as in the previous experiment, there were no significant differences between the groups due to differences in dietary composition. Values increased significantly in the NP+M and LP+M+T groups after LPS treatment compared with the corresponding pair-fed controls. A trend for an increase in the relative liver protein was found after LPS treatment compared with the pair-fed animals in the VLP+M+T group, but this increase was not statistically significant (Table 6.9).

Comparison of data from the present study, with that reported in the previous chapter, indicates that addition of tryptophan to the low-protein dietary groups had no effect on the relative liver protein during the inflammatory response (see Table 5.9, chapter 5 & Table 6.9).

Table 6.9 Protein in liver and lung of rats fed diets of graded casein content but with identical amounts of sulphur amino acids and tryptophan, after starting treatments

Dietary groups g protein/kg diet	NP+M 180+M	LP+M+T 80+M+T	VLP+M+T 60+M+T	two-way ANOVA p-value	F ratio
Protein in liver (mg/g liver)					
ad libitum	151.1 ± 2.8 [a]	136.8 ± 4.0 [b]	124.0 ± 5.4 [b]	D=0.001	F=8.36
pair-fed	160.6 ± 2.2 [a]*	152.0 ± 7.8 [a]	142.5 ± 10.0 [a]	T=0.046	F=3.34
LPS	161.7 ± 2.8 [a]*	145.5 ± 4.6 [b]	134.6 ± 9.6 [b]	I=0.968	F=0.14
Total liver protein (mg/ total liver)					
ad libitum	876 ± 44 [ab]	924 ± 57 [a]	764 ± 37 [b]	D=0.001	F=9.01
pair-fed	740 ± 33 [a]	667 ± 33 [ab]*	585 ± 35 [b]*	T=0.000	F=12.69
LPS	822 ± 46 [a]	749 ± 68 [ab]	633 ± 34 [b]*	I=0.686	F=0.57
Relative liver protein (g/kg body weight)					
ad libitum	7.30 ± 0.36 [a]	8.62 ± 0.24 [a]	8.39 ± 0.21 [a]	D=0.085	F=2.63
pair-fed	7.11 ± 0.17 [a]	7.15 ± 0.24 [a] *	7.10 ± 0.17 [a] *	T=0.000	F=13.61
LPS	8.21 ± 0.36 [a] ♠	8.46 ± 0.27 [a] ♠	7.92 ± 0.35 [a]	I=0.103	F=2.08
Lung protein (mg/g lung)					
ad libitum	72.5 ± 1.2 [a]	71.8 ± 1.3 [a]	73.8 ± 2.0 [a]	D=0.776	F=0.26
pair-fed	72.5 ± 1.7 [a]	73.5 ± 1.5 [a]	71.5 ± 1.0 [a]	T=0.299	F=1.25
LPS	75.9 ± 1.0 [a]	73.2 ± 0.7 [a]	73.4 ± 1.3 [a]	I=0.436	F=0.97
Total lung protein (mg/ total lung)					
ad libitum	64.5 ± 1.3 [a]	63.1 ± 3.5 [a]	56.8 ± 3.6 [a]	D=0.000	F=11.97
pair-fed	65.5 ± 4.3 [a]	57.9 ± 2.2 [b]	51.0 ± 2.1 [c]	T=0.057	F=3.1
LPS	62.8 ± 3.0 [a]	54.8 ± 2.1 [a b]	48.7 ± 2.9 [b]	I=0.755	F=0.47
Relative lung protein (mg/kg body weight)					
ad libitum	0.54 ± 0.02 [a]	0.59 ± 0.02 [a]	0.63 ± 0.05 [a]	D=0.728	F=0.32
pair-fed	0.63 ± 0.04 [a]	0.63 ± 0.02 [a]	0.63 ± 0.04 [a]	T=0.256	F=1.41
LPS	0.63 ± 0.03 [a]	0.63 ± 0.04 [a]	0.61 ± 0.02 [a]	I=0.598	F=0.70

-Results are presented as mean ± standard error of the mean, n=4 per treatment group for animals fed the NP+M diet, and n=6 per treatment group for animals fed the LP+M+T and the VLP+M+T diets. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

d) Lung protein concentration

As in the previous experiment, animals were able to maintain lung protein concentration even under conditions of protein deficiency. No significant difference in lung protein concentration was found due to diet or treatments between the groups (Table 6.9).

e) Total lung protein

In *ad libitum*-fed animals, there was a trend for a reduction in the total lung protein content as dietary protein content was decreased, but this difference was not statistically significant (Table 6.9). In the pair-fed animals, there was a progressive reduction in total lung protein as dietary protein content was decreased. In the LPS-treated animals, there was a trend for a reduction in total lung protein as dietary protein content was decreased. This was statistically significant when comparing the NP+M group with the VLP+M+T group.

No difference in total lung protein was found due to treatments as a factor.

f) Total lung protein/kg body weight

As in the previous study, the total lung protein, as a proportion of body weight, was maintained at the same level in all animals of various diet and treatment groups. No significant difference between the groups was found due to diet or treatments (see Table 5.9, chapter 5 & Table 6.9).

6.4.4 Serum concentrations of α-1 acid glycoprotein and albumin

α-1 acid glycoprotein

As in the previous experiment, endotoxin-treated rats significantly increased the acute phase protein, α-1 acid glycoprotein, compared with *ad libitum* and pair-fed animals (Table 6.10). In the LPS-treated groups, addition of tryptophan to the animals fed the low-protein diets supplemented with methionine (the LP+M+T and VLP+M+T groups) resulted in a lower mean values compared with the low-protein

diets supplemented with methionine alone (the LP+M and VLP+M groups). The serum concentration of α-1 acid glycoprotein reduced from 931 and 946 μg/ml for the LP+M and VLP+M groups respectively to 777 and 790 μg/ml for the LP+M+T and VLP+M+T groups respectively. However this reduction was not statistically significant (See Table 5.10; chapter 5 & Table 6.10).

Table 6.10 Serum α-1 acid glycoprotein and albumin of rats fed diets of graded casein content but with identical amounts of sulphur amino acids and tryptophan, after starting treatments

Dietary groups g protein/kg diet	NP+M 180+M	LP+M+T 80+M+T	VLP+M+T 60+M+T	two-way ANOVA p-value	 F ratio
α-1 acid glycoprotein (μg/ml)					
ad libitum	86 ± 14 [a]	84 ± 7 [a]	87 ± 9 [a]	D=0.720	F=0.33
pair-fed	99 ± 8 [a]	90 ± 9 [a]	84 ± 5 [a]	T=0.000	F=244.9
LPS	854 ± 71 [a]♠*	777 ± 82 [a]♠*	790 ± 75 [a]♠*	I=0.937	F=0.20
Albumin (mg/ml)					
ad libitum	35.8 ± 0.22 [a]	32.2 ± 0.60 [b]	29.4 ± 0.39 [c]	D=0.000	F=49.73
pair-fed	35.7 ± 0.87 [a]	32.9 ± 0.76 [a]	30.3 ± 0.81 [b]	T=0.003	F=6.63
LPS	34.3 ± 1.17 [a]	30.0 ± 0.58 [b]♠*	28.7 ± 0.49 [b]	I=0.689	F=0.57

-Results are presented as mean ± standard error of the mean, n=4 per treatment group for animals fed the NP+M diet, and n=6 per treatment group for animals fed the LP+M+T and the VLP+M+T diets. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

Albumin

In all dietary groups, feeding diets severely deficient in protein decreased serum albumin concentration (Table 6.10). The endotoxin-treated animals had a slightly lower albumin than the corresponding pair-fed animals. This difference was statistically significant in animals fed the LP+M+T diet. Addition of tryptophan to the animals fed the low-protein diets supplemented with methionine did not exert any effect on serum albumin concentration (See Table 5.10, chapter 5 & Table 6.10).

6.4.5 Inorganic sulphate excretion, and urinary nitrogenous end products of amino acid metabolism: Urea-N and ammonia-N excretion

Inorganic sulphate excretion, and inorganic sulphate excretion as a percentage of sulphur amino acid intake-average of two days before starting treatments

No difference in inorganic sulphate (SO_4) excretion was found between the groups, before starting treatments. However, animals fed the very low-protein diet had the highest SO_4 excretion, when it expressed as a percentage of sulphur amino acid intake. A similar phenomenon was noted in the previous experiment (See Table 5. 11, chapter 5 & Table 6.11).

Table 6.11 Inorganic sulphate excretion (SO_4), and inorganic sulphate excretion as a percentage of sulphur amino acid intake (S.A.A.I.) of rats fed diets of graded casein content but with identical amounts of sulphur amino acids and tryptophan-average of two days before starting treatments (µmol/day)

Dietary groups	NP+M	LP+M+T	VLP+M+T	one-way ANOVA
g protein/kg diet	180+M	80+M+T	60+M+T	p-value F ratio
SO_4 excretion	302 ± 17 [a]	321 ± 13 [a]	341 ± 17 [a]	p=0.239 F=1.50
(SO_4/S.A.A.I.) x100	34.8 ± 1.5[a]	36.0 ± 1.8 [a]	42.1 ± 1.4 [b]	p=0.005 F=6.23

-Results are presented as mean ± standard error of the mean, n=12 per group. Means within each row having different letter superscripts following the number differ significantly.

Inorganic sulphate excretion after starting treatments

The results of SO_4 excretion, after starting treatments, can be seen in Table 6.12. In animals fed *ad libitum*, no significant difference in SO_4 excretion was found between the groups. The mean values decreased in the LPS treated and in pair-fed animals compared with the *ad libitum*-fed animals. This decrease was statistically significant when comparing the pair-fed animals with the corresponding animals fed the NP+M and LP+M+T diets *ad libitum*. Comparing the LPS-treated rats with the pair-fed controls, the mean values for SO_4 excretion increased in the NP+M group after LPS treatment, but because of the wide inter-group variability, this increase was not statistically significant. In the two low-protein dietary groups

(the LP+M+T and VLP+M+T groups), no difference in SO_4 excretion was found, when comparing the LPS-treated rats with the pair-fed controls.

Table 6.12 Inorganic sulphate excretion of rats fed diets of graded casein content but with identical amounts of sulphur amino acids and tryptophan, after starting treatments (μmol/day)

Dietary groups	NP+M	LP+M+T	VLP+M+T	two-way ANOVA	
g protein/kg diet	180+M	80+M+T	60+M+T	p-value	F ratio
ad libitum	316 ± 2 [a]	314 ± 14 [a]	338 ± 16 [a]	D=0.015	F=4.94
pair-fed	182 ± 36 [a]*	192 ± 20 [a]*	298 ± 15 [b]	T=0.001	F=9.97
LPS	236 ± 46 [a]	196 ± 46 [a]	277 ± 35 [a]	I=0.463	F=0.93

-Results are presented as mean ± standard error of the mean, n=4 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

Ammonia-N and urea-N excretion-average of two days before starting treatments

A significant reduction in NH_3-N and urea-N excretion was found in the LP+M+T and VLP+M+T group compared with the NP+M group. No difference in NH_3-N and urea-N excretion was found between the LP+M+T and the VLP+M+T groups (Table 6.13).

Urea-N excretion was slightly increased (not statistically significant) after addition of tryptophan to the very low-protein diet supplemented with methionine (the VLP+M+T group) when compared with animals fed the same diet, but without tryptophan supplementation in the previous experiment (see Table 5.13, chapter 5 & Table 6.13).

Table 6.13 Ammonia-N and urea-N excretion of rats fed diets of graded casein content but with identical amounts of sulphur amino acids and tryptophan -average of two days before starting treatments (µmol/day)

Dietary groups g protein/kg diet	NP+M 180+M	LP+M+T 80+M+T	VLP+M+T 60+M+T	one-way ANOVA p-value	 F ratio
NH_3-N	789 ± 26 [a]	350 ± 22 [b]	349 ± 21 [b]	p=0.000	F=123.36
Urea-N	9507 ± 307 [a]	815 ± 44 [b]	772 ± 52 [b]	p= 0.000	F=672.99

-Results are presented as mean ± standard error of the mean, n=12 per group. Means within each row having different letter superscripts following the number differ significantly.

Ammonia-N and urea-N excretion after starting treatments

a) Ammonia-N excretion after starting treatments

Table 6.14 shows ammonia-N excretion, after starting treatments. In animals fed *ad libitum*, LPS-treated rats, and pair-fed controls, a significant reduction in NH_3-N excretion occurred in the LP+M+T and VLP+M+T groups when compared with the NP+M group. No difference in NH_3-N and urea-N excretion was found between the LP+M+T and VLP+M+T groups.

Due to treatments, in all the groups, the mean values of NH_3-N excretion was lower in the LPS treated and pair-fed animals compared with animals fed *ad libitum*, but this difference was statistically significant, when comparing the pair-fed animals with the corresponding *ad libitum*-fed animals given the NP+M diet. Addition of tryptophan did not appear to modify the pattern of response (see Table 5.14, chapter 5 & Table 6.14).

Table 6.14 Ammonia-N and urea-N excretion of rats fed diets of graded casein content but with identical amounts of sulphur amino acids and tryptophan, after starting treatments (μmol/day)

Dietary groups g protein/kg diet	NP+M 180+M	LP+M+T 80+M+T	VLP+M+T 60+M+T	two-way p-value	ANOVA F ratio
NH_3-N					
ad libitum	752 ± 25 [a]	349 ± 65 [b]	319 ± 20 [b]	D=0.000	F=43.58
pair-fed	462 ± 61 [a]*	190 ± 30 [b]	266 ± 11 [b]	T=0.000	F=10.24
LPS	539 ± 88 [a]	176 ± 34 [b]	269 ± 54 [b]	I=0.194	F=1.64
Urea-N					
ad libitum	10134 ± 530 [a]	727 ± 66 [b]	831 ± 109 [b]	D=0.000	F=307.50
pair-fed	6211 ± 449 [a]*	537 ± 52 [b]	637 ± 40 [b]	T=0.000	F=10.92
LPS	6970 ± 1127 [a]	1129 ± 262 [b]	912 ± 206 [b]	I=0.000	F=9.67

-Results are presented as mean ± standard error of the mean, n=4 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

b) Urea-N excretion after starting treatments

The results of urea-N excretion, after starting treatments, can be seen in Table 6.14. In the *ad libitum*, LPS treated and pair-fed animals, there was a significant reduction in the amounts of urea-N excretion occurred in the LP+M+T and VLP+M+T groups when compared with the NP+M group. No difference in urea-N excretion was found between the LP+M+T and the VLP+M+T groups.

Due to treatments, in the NP+M group, pair-fed animals excreted lower amounts of urea-N than animals fed *ad libitum*. In all the groups, the mean values of urea-N excretion were increased after LPS treatment when compared with the pair-fed animals, but this effect was not statistically significant.

Although the food intake was similar between the LPS-treated animals, fed the VLP+M+T diet in the present study, and LPS-treated animals, fed the same diet but without added tryptophan in the previous study, urea-N excretion increased by 32% after inclusion of tryptophan in the diet (see Table 5.14, chapter 5 & Table 6.14).

6.4.6 Relationship between parameters measured in the study

The ratio of glutathione to protein

Dietary protein content, food intake and LPS treatment caused a significant alteration in the ratio of GSH to protein (Table 6.15).

In the *ad libitum* and in pair-fed animals, the ratio of GSH to protein was significantly higher in the VLP+M+T group compared with the NP+M and the LP+M+T groups. In the LPS-treated animals, the ratio of GSH to protein was significantly higher in the VLP+M+T group compared with the NP+M group. The mean value was higher in the VLP+M+T group compared with the LP+M+T group, but the difference was not statistically significant.

Due to treatments, in all dietary groups, the ratio was significantly higher after LPS treatment compared with the corresponding pair-fed animals. In all dietary groups, pair feeding caused a significant reduction in the ratio compared to values for *ad libitum*-fed animals.

Comparison of data from the present study with that of the previous study, it can be seen that addition of tryptophan to the low-protein diets supplemented with methionine (the LP+M+T and VLP+M+T diets) appeared to increase (not statistically significant) the ratio of GSH to protein present in liver, in the LPS-treated animals and in animals fed *ad libitum*, when compared with animals fed the same diets, but without tryptophan supplementation (the LP+M and VLP+M diets). The increase in the ratio was greatest in animals fed the VLP+M+T diet (see Table 5.15, chapter 5 & Table 6.15).

Table 6.15 The ratio of hepatic glutathione to hepatic protein and to inorganic sulphate excretion (SO_4) of rats fed diets of graded casein content but with identical amounts of sulphur amino acids and tryptophan, after starting treatments

Dietary groups g protein/kg diet	NP+M 180+M	LP+M+T 80+M+T	VLP+M+T 60+M+T	two-way ANOVA p-value F ratio
GSH/ protein (nmol/mg)				
ad libitum	42.4 ± 1.2 ^{a}	47.5 ± 5.4 ^{a}	74.1 ± 7.5 ^{b}	D=0.000 F=27.42
pair-fed	23.4 ± 3.6 ^{a}*	15.0 ± 1.8 ^{a}*	40.1 ± 1.8 ^{b}*	T=0.000 F=43.30
LPS	46.6 ± 1.6 ^{a}♠	53.1 ± 1.5 ^{b}♠	67.7 ± 5.7 ^{b}♠	I=0.264 F=1.37
GSH/SO_4 (nmol per liver /µmol per day)				
ad libitum	118 ± 9^{a}	151 ± 11 $^{a\,b}$	178 ± 12^{b}	D=0.593 F=0.53
pair-fed	98 ± 9 ^{a}	59 ± 8 ^{b}*	78 ± 4 $^{a\,b}$*	T=0.000 F=41.50
LPS	174 ± 22 ^{a} ♠	214 ± 16 ^{a} ♠ *	164 ± 25 ^{a} ♠	I=0.006 F=4.52

-Results are presented as mean ± standard error of the mean. For the ratio of hepatic glutathione to hepatic protein, n=4 per treatment group for animals fed the NP+M diet, and n=6 per treatment group for animals fed the LP+M+T and the VLP+M+T diets. For the ratio of hepatic glutathione to inorganic sulphate excretion, n=4 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

The ratio of total glutathione in liver to inorganic sulphate excreted in urine after starting treatments

Table 6.15 shows the ratio of total glutathione in liver to SO_4 excreted in urine. In animals fed *ad libitum*, there was an increase in the ratio of total hepatic GSH to SO_4 excreted in urine as dietary protein content was decreased, and this increase in the ratio was statistically significant when comparing the VLP+M+T group with the NP+M group. In the pair-fed controls, the ratio in the LP+M+T group was lower than in the NP+M group. In the LPS-treated animals, no significant differences were found between the groups.

Comparing the LPS-treated rats with the pair-fed controls, the ratio was significantly increased in all dietary groups after LPS treatment.

All dietary groups were able to increase the ratio of glutathione in liver to SO_4 excreted in urine after LPS treatment, as in the last study. Despite the similarity in the amount of food eaten (and the similarity in sulphur amino acid intake) between LPS-treated animals fed the VLP+M+T diet in the present study and animals fed the same diet, but without tryptophan supplementation in the previous study (the LPS-treated animals fed the VLP+M diet), the ratio of hepatic glutathione to urinary SO_4 excretion slightly increased (not statistically significant) by addition of tryptophan to the diet (see Table 5.15, chapter 5 & Table 6.15).

The ratio of ammonia-N and urea-N excretion to N intake before starting treatment

When NH_3-N excretion correlated to N intake, it was found that the ratio of NH_3-N excretion to N intake was significantly higher in the VLP+M+T group compared to other groups. There was a significant reduction in the ratio of urea-N excretion to N intake in the LP+M+T and VLP+M+T groups compared with the NP+M group. The ratio of urea-N excretion to N intake was significantly higher in VLP+M+T group than in the LP+M+T group (Table 6.16).

Addition of tryptophan had no effect on the ratio of ammonia-N to N intake, but it significantly increased the ratio of urea-N excretion to N intake in animals fed the VLP+M+T diet in the present study compared with animals fed the same diet, but without tryptophan supplementation in the previous chapter (see Table 5.16; chapter 5 & Table 6.16) .

Table 6.16 The ratio of ammonia-N and urea-N excretion to N intake of rats fed diets of graded casein content but with identical amounts of sulphur amino acids and tryptophan-average of two days before starting treatments (mg/mg)

Dietary groups	NP+M	LP+M+T	VLP+M+T	one-way ANOVA	
g protein/kg diet	180+M	80+M+T	60+M+T	p-value	F-ratio
NH_3-N/N intake	0.029 ± 0.001[a]	0.026 ± 0.002[a]	0.038 ± 0.002[b]	p=0.000	F=11.9
Urea-N/N intake	0.350 ± 0.022[a]	0.064 ± 0.003[b]	0.083 ± 0.004[c]	p=0.000	F=138.5

* Results are presented as mean ± standard error of the mean, n=12 per group. Means within each row having different letter superscripts following the number differ significantly.

The ratio of ammonia-N and urea-N excretion in urine to N intake after starting treatments

a) The ratio of ammonia-N excretion in urine to N intake after starting treatments

Dietary protein content caused a significant alteration on the ratio of NH_3-N to N intake (Table 6.17). In animals fed *ad libitum*, there was a significant increase in the ratio of NH_3-N to N intake in the VLP+M+T group compared with the NP+M group. The ratio was also higher in the VLP+M+T group than in the LP+M+T group, but this increase in the ratio was not statistically significant. In the LPS-treated animals and in pair-fed animals no significant difference in the ratio was found between the groups.

Table 6.17 The ratio of ammonia-N and urea-N excretion in urine to N intake of rats fed diets of graded casein content but with identical amounts of sulphur amino acids and tryptophan, after starting treatments (mg/mg)

Dietary groups g protein/kg diet	NP+M 180+M	LP+M+T 80+M+T	VLP+M+T 60+M+T	two-way ANOVA p-value F ratio
NH_3-N/N intake				
ad libitum	0.024 ± 0.0003 [a]	0.025 ± 0.003 [ab]	0.032 ± 0.002 [b]	D=0.033 F=10.02
pair-fed	0.040 ± 0.012 [a]	0.038 ± 0.008 [a]	0.038 ± 0.002 [a]	T=0.118 F=1.22
LPS	0.061 ± 0.019 [a]	0.044 ± 0.010 [a]	0.036 ± 0.005 [a]	I=0.502 F=0.76
Urea-N/N intake				
ad libitum	0.324 ± 0.006 [a]	0.056 ± 0.004 [b]	0.082 ± 0.008 [c]	D=0.000 F=19.52
pair-fed	1.052 ± 0.372 [a]	0.118 ± 0.029 [a]	0.090 ± 0.008 [a]	T=0.007 F=6.15
LPS	1.148 ± 0.437 [a]	0.297 ± 0.082 [a]	0.107 ± 0.006 [a]*	I=0.083 F=2.33

-Results are presented as mean ± standard error of the mean, n=4 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

b) The ratio of urea-N excretion in urine to N intake after starting treatments

In animals fed *ad libitum*, a significant reduction in the ratio of urea-N excretion to N intake was found in the low-protein dietary groups (the LP+M+T and VLP+M+T groups) when compared with the NP+M group. However, values for the

VLP+M+T group were significantly higher than those for the LP+M+T group. No significant difference in the ratio was found between the LPS-treated animals and pair-fed animals (Table 6.17). The appetite response to LPS injection in the LPS-treated animals within the same dietary group varied considerably which made the standard deviation of the mean of protein intake after LPS treatment high, similarly for the pair-fed animals. Consequently, the difference was not statistically significant. However, it is clear that the mean values of the ratio in the LPS-treated animals and pair-fed animals fed the NP+M diet was dramatically higher than in the LPS-treated animals and pair-fed animals fed the LP+M+T and VLP+M+T diets.

Due to treatments, the ratio of urea-N to N intake, in the VLP+M+T group, was significantly higher in the LPS-treated animals than in the *ad libitum*-fed animals.

Addition of tryptophan to the very low-protein diet supplemented with methionine (the VLP+M+T group) increased (not statistically significant ($p<0.068$)) the ratio of urea-N excretion to N intake in the *ad libitum*-fed animals, but not in the LPS-treated rats or pair-fed animals when compared to values from animals fed the same diet, but without tryptophan supplementation in the previous study (see Table 5.17; chapter 5 & Table 6.17).

The ratio of inorganic sulphate excretion to ammonia-N and urea-N excretion before starting treatment

a) The ratio of inorganic sulphate excretion to ammonia-N excretion before starting treatments

The ratio of SO_4 to NH_3-N excretion in urine was significantly higher in the low-protein dietary groups than in the NP+M group. No difference in the ratio was found between the LP+M+T group and the VLP+M+T group (Table 6.18).

Addition of Tryptophan to the low-protein diets supplemented with methionine did not influence the ratio (see Table 5.18, chapter 5 &Table 6.18).

b) The ratio of inorganic sulphate excretion to urea-N excretion before starting treatments

The ratio of SO_4 to urea-N excretion in urine was significantly higher in the low-protein dietary groups than in the NP+M group. No significant difference in the ratio was found between the LP+M+T group and the VLP+M+T group (Table 6.18).

The ratio of SO_4 to urea-N excretion in the VLP+M+T group was slightly decreased (not statistically significant) compared with animals fed the same diet, but without added tryptophan in the last study (see Table 5.18, chapter 5 & Table 6.18).

Table 6.18 The ratio of inorganic sulphate excretion (SO_4) to ammonia-N and urea-N excretion of rats fed diets of graded casein content but with identical amounts of sulphur amino acids and tryptophan -average of two days, before starting treatments (μmol/μmol)

Dietary groups g protein/kg diet	NP+M 180+M	LP+M+T 80+M+T	VLP+M+T 60+M+T	one-way ANOVA p-value	 F ratio
SO_4/NH_3-N	0.38 ± 0.02^a	0.89 ± 0.02^b	0.99 ± 0.04^b	p=0.000	F =135.36
SO_4/Urea-N	0.032 ± 0.002^a	0.391 ± 0.029^b	0.456 ± 0.028^b	p=0.000	F=117.08

-Results are presented as mean ± standard error of the mean, n=12 per group. Means within each row having different letter superscripts following the number differ significantly.

The ratio of inorganic sulphate excretion to ammonia-N and urea-N excretion after starting treatments

a) The ratio of inorganic sulphate excretion to ammonia-N after starting treatments

The results of the ratio of inorganic sulphate excretion to ammonia-N excretion, after starting treatments, can be found in Table 6.19. In the *ad libitum*, LPS treated and pair-fed animals, the ratio of SO_4 to NH_3-N excretion in urine was significantly higher in the low-protein dietary groups than in the NP+M group. No significant difference in the ratio was found between the LP+M+T group and the

VLP+M+T group. The ratio was not affected by LPS treatment or restriction in food intake.

Despite a similarity in the amount of food eaten (and therefore sulphur amino acid intake) between LPS-treated animals fed the VLP+M+T diet in the present study and LPS-treated animals fed the same diet, but without added tryptophan in the previous study, the ratio of SO_4 to ammonia-N excretion was decreased after inclusion of tryptophan in the diet, however the difference did not reach statistical significance (see Table 5.19, chapter 5 & Table 6.19).

Table 6.19 The ratio of inorganic sulphate excretion (SO_4) to ammonia-N and urea-N excretion of rats fed diets of graded casein content but with identical amounts of sulphur amino acids and tryptophan, after starting treatments (μmol/μmol)

Dietary groups g protein/kg diet	NP+M 180+M	LP+M+T 80+M+T	VLP+M+T 60+M+T	two-way ANOVA p-value	 F ratio
SO_4/NH_3-N					
ad libitum	0.42 ± 0.01 [a]	0.99 ± 0.17 [b]	1.07 ± 0.07 [b]	D=0.000	F=49.62
pair-fed	0.39 ± 0.02 [a]	1.05 ± 0.09 [b]	1.12 ± 0.06 [b]	T=0.741	F=0.30
LPS	0.44 ± 0.04 [a]	1.12 ± 0.12 [b]	1.09 ± 0.13 [b]	I=0.938	F=0.20
SO_4/urea-N					
ad libitum	0.032 ± 0.002 [a]	0.445 ± 0.049 [b]	0.430 ± 0.061 [b]	D=0.000	F=61.29
pair-fed	0.024 ± 0.002 [a]	0.362 ± 0.026 [b]	0.473 ± 0.037 [b]	T=0.002	F=8.22
LPS	0.029 ± 0.004 [a]	0.176 ± 0.009 [b] ♠*	0.355 ± 0.052 [c]	I=0.016	F=3.73

-Results are presented as mean ± standard error of the mean, n=4 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

b) The ratio of inorganic sulphate excretion to urea-N after starting treatments

The results of the ratio of inorganic sulphate excretion to urea-N excretion can be found in Table 6.19. In animals fed *ad libitum*, the ratio of SO_4 excretion to urea-N excretion was significantly higher in the low-protein dietary groups compared with the NP+M group. No significant difference in the ratio was found between the low-protein groups. In the pair-fed animals, the ratio was significantly lower in the NP+M group compared with other groups, and the mean value of the

ratio was higher in the VLP+M+T group compared with the LP+M+T group, but this difference was not statistically significant. In LPS-treated animals, a significant increase in this ratio was found as the protein concentration in the diet was decreased. However, the magnitude of the increase was much higher when comparing animals fed the NP+M diet with animals fed the low-protein diets, than when comparing animals fed the LP+M+T diet with animals fed the VLP+M+T diet.

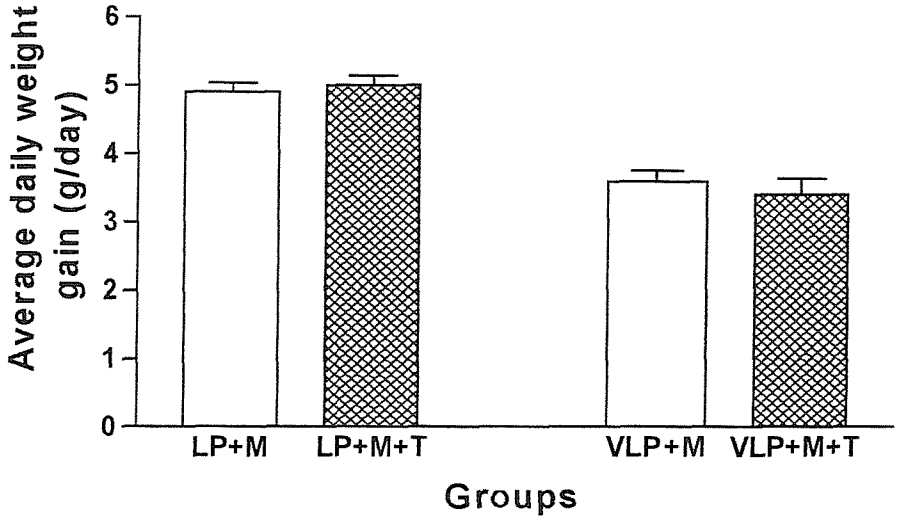
So the main finding is that in all treatment groups, the ratio increases as protein intake falls. However, it can be seen that inflammation partially corrects the increase caused by a fall in protein intake in the low-protein groups.

In *ad libitum*-fed animals, the ratio of SO_4 to urea-N excretion in animals fed the VLP+M+T diet was slightly decreased (not statistically significant) compared with animals fed the same diet but without added tryptophan, in the last study (see Table 5.19, chapter 5 & Table 6.19). Otherwise, addition of tryptophan to the low-protein dietary groups did not exert any effect on the pattern of the response.

6.5 Discussion

Addition of tryptophan to the low-protein diets containing adequate amount of methionine did not improve growth to a greater extent than methionine supplementation alone (see Figure 6-4). This is because tryptophan is not the most limiting amino acid for growth in animals fed the low-protein diets (see Table 4.2, chapter 4). The most limiting amino acid is methionine, in which one-third to one-half of methionine requirement can be supplied by L-cystine (National Research Council, 1978).

Figure 6-4 Average daily weight gain of animals fed low-protein diets supplemented with methionine and tryptophan compared with animals fed the same diets, but without tryptophan supplementation



- Results are means ± SE. N=18 per group.

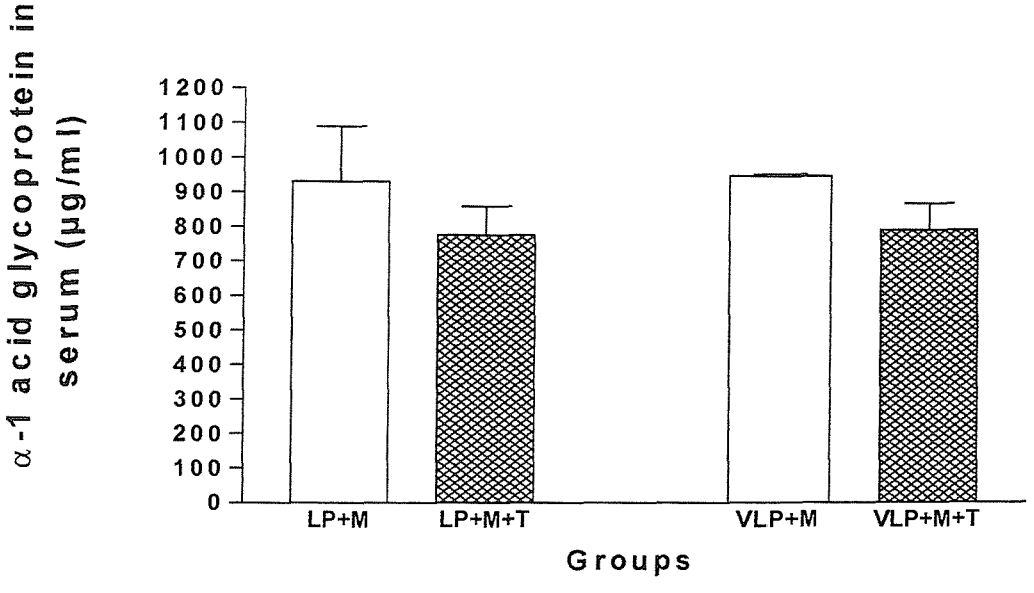
It was hypothesised by Reeds *et al.* (1994) that the imbalance between the proportions of amino acids released from muscle and the proportions needed for acute phase protein synthesis, was responsible for the negative nitrogen balance during the inflammatory response. Reeds *et al.* (1994) calculated that tryptophan and other aromatic amino acids were important during the response. The results of the present study do not support either of these concepts. There was no indication that addition of tryptophan had any effect in enhancing the acute inflammatory response

after exposure to LPS. Tryptophan intake would instead appear to be present in excess of the requirement for inflammatory response in the present study.

After exposure to the acute inflammatory effects of LPS, liver, lung, spleen and thymus relative weights, in animals fed the low-protein diets supplemented with methionine and tryptophan (the LP+M+T and VLP+M+T groups), were not increased further compared with animals fed the same diets, but with only methionine supplementation in the previous study (see Table 5.5, chapter 5 & Table 6.5).

Whichever way liver protein is expressed (e.g. concentration, total protein or relative to body weight), there was no evidence that addition of tryptophan to the low-protein dietary groups supplemented with methionine had any stimulatory on the hepatic protein metabolism (see Table 5.9; chapter 5 & Table 6.9). Furthermore, α-1 acid glycoprotein, one of the major acute phase protein in rats, was not enhanced further in the LPS-treated animals after addition of tryptophan to the low and very low-protein diets supplemented with methionine, indeed, the data suggested that addition of tryptophan might even suppress this aspect of the response to LPS (Figure 6-5).

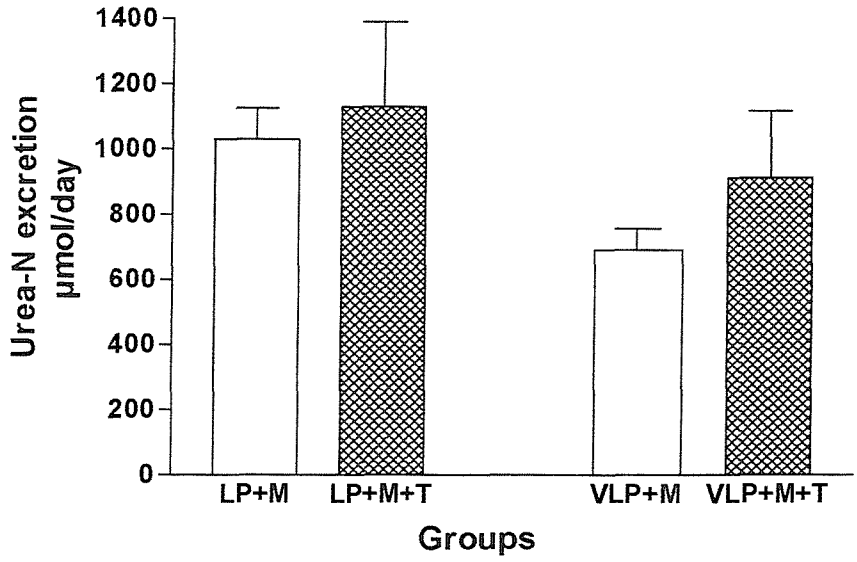
Figure 6-5 α-1 acid glycoprotein in LPS-treated animals fed low-protein diets supplemented with methionine and tryptophan compared with animals fed the same diets, but without tryptophan supplementation



- Results are means ± SE. N=6 per treatment group.

Despite a similarity in food intake between the VLP+M+T group in the present study and the VLP+M group in the previous study, after exposure to the inflammatory effects of LPS, urea-N excretion was greater after addition of tryptophan and methionine to the diet than when methionine was added alone, but this increase was not statistically significant (Figure 6-6). This finding again suggests that tryptophan is not a limiting amino acid for acute phase response in animals fed a severely protein restricted diet, as an improvement in nitrogen balance would be expected if Reeds *et al.*'s hypothesis was correct.

Figure 6-6 Urea-N excretion of LPS-treated animals fed low-protein diets supplemented with methionine and tryptophan compared with animals fed the same diets, but without tryptophan supplementation



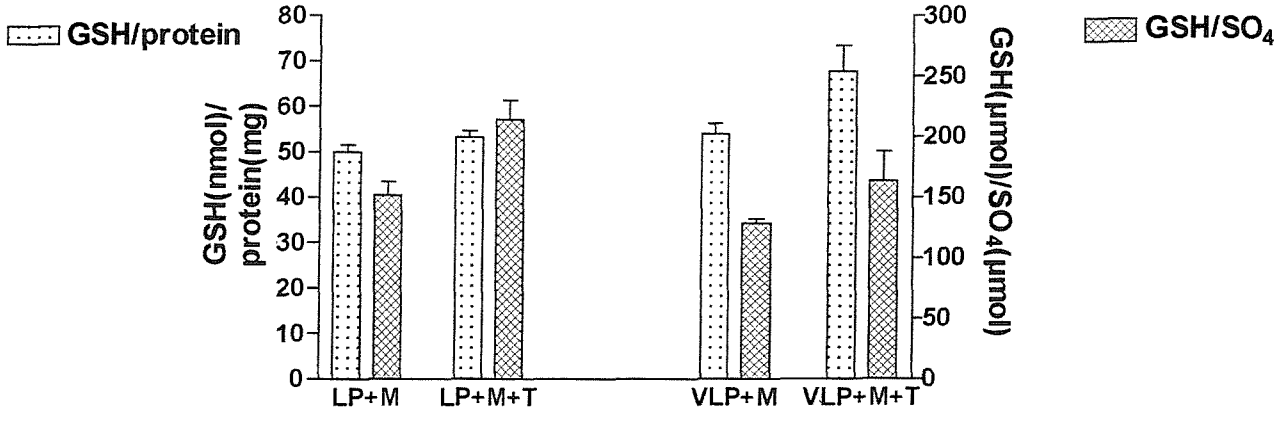
- Results are means ± SE. N=4 per treatment group.

While addition of tryptophan did not appear to increase the magnitude of the acute phase response, it appeared to change the partitioning of sulphur amino acids between the main end products, protein, GSH and SO_4.

The ratio of glutathione to protein in liver, and the ratio of hepatic glutathione to SO_4 excretion were increased (not statistically significant) in the LPS-treated animals and also in the *ad libitum*-fed animals, after addition of tryptophan to the

low-protein diets supplemented with methionine (see Tables 5.15; chapter 5 & Table 6.15). This effect can be seen most clearly in the LPS-treated animals fed the VLP+M+T diet (Figure 6-7). The food intake of LPS-treated animals fed the VLP+M+T group in the present study and the food intake of LPS-treated animals fed the VLP+M group in the previous study was similar, thus sulphur amino acid intake is not a variable influencing the increase in the ratio. Since, SO_4 excretion, the amount of weight gain and the relative liver protein were similar between the above groups, it could be postulated that the increase in the ratio of GSH to protein present in liver and the increase in the ratio of hepatic GSH to SO_4 excretion in the present study, after addition of tryptophan, could be due to the possibility that the additional tryptophan had improved the supply of glycine and hence partitioned more cysteine into glutathione synthesis. Such an explanation is biochemically feasible.

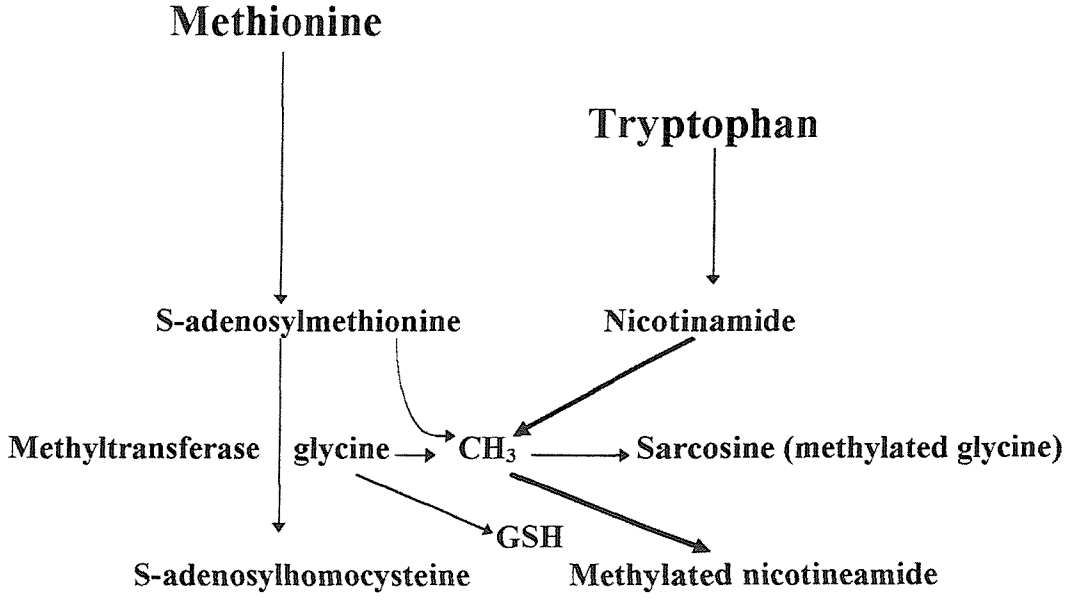
Figure 6-7 The ratio of glutathione to protein present in liver, and the ratio of hepatic glutathione to inorganic sulphate excretion of the LPS-treated animals fed the low-protein diets supplemented with methionine and tryptophan compared with animals fed the same diets, but without tryptophan supplementation



-Results are means ± SE. n=6 per treatment group for ratio of glutathione to protein present in liver, and n=4 for the ratio of hepatic glutathione to inorganic sulphate excretion.

The conversion of S-adenosylmethionine to S-adenosylhomocysteine requires the removal of a methyl group. Normally glycine acts as the methyl acceptor. However, nicotinamide, which is a major product of tryptophan metabolism, can also act as a methyl acceptor forming N-methyl nicotinamide and methyl pyridone carboxyamide, hence reducing the requirement for the methylation of glycine. Consequently, more glycine will be available for glutathione synthesis (see Figure 6-8). In an in vivo study, which increased tryptophan intake to a greater extent than in the present study, (from 1.9 g to 5.9 g/kg diet compared with the present study, from 1 g and 0.7 g/kg diet for the LP and VLP diets respectively to 2.2 g/kg diet for both the LP+M+T and VLP+M+T diets), a considerable increase in the metabolites of nicotinamide, N-methylnicotinamide and methyl pyridone nicotinamide occurred (McCreanor & Bender, 1986).

Figure 6-8 The possible role of tryptophan in conserving glycine for glutathione synthesis



From the work presented in this study and in the previous chapter it would seem that in many respects, the requirement for growth and for the inflammatory response was somewhat similar. Addition of methionine to the low-protein groups, in the previous chapter (chapter 5), increased the weight gain of rats, and at the same time enhanced the inflammatory response, as indicated by the increase in GSH concentration in various organs, in which the increase was dramatic in liver. In addition, liver protein content and α-1 acid glycoprotein were improved after addition of methionine to the low-protein groups. However, addition of tryptophan to the low-protein dietary groups supplemented with methionine, did not improve the weight gain of rats and at the same time did not exert major effect on the inflammatory response, when compared with animals fed the same diets, but with only methionine supplementation.

It could be argued that the failure to demonstrate that tryptophan is a limiting amino acid in the acute phase response may relate to species differences in amino acid requirement of humans, for whom Reeds *et al.* (1994) prepared there hypothesis, and rats, used as a model of inflammation in the present study. If, however, we look to the proportion of nine essential amino acids needed to support the growth in young rat and compare it with the proportion of these amino acids needed to support growth in children (Table 6.20), we can see that the requirements between the two species are somewhat different. The requirement for sulphur amino acids and aromatic amino acids are higher in young rats than in children. The requirement for valine was similar between the two species. Finally, the requirement for the remaining amino acids was higher in children than in young rats. The requirement for tryptophan for growth is however markedly higher in rats than in children. If the amino acid requirement for growth and inflammation were proportionately similar, an effect from tryptophan supplementation would have been expected in the present study.

Table 6.20 The proportion of nine essential amino acids needed to support growth in young rats compared with the proportion of these amino acids needed to support growth in children

Amino acid	young rats* %	schoolchildren 10-12 years • %
Histidine	6.1	0
Isoleucine	10.2	11.7
Leucine	15.3	17.2
Lysine	14.3	23
Methionine + cystine	12.2	10.4
Phenylalanine + tyrosine	16.3	10.4
Threonine	10.2	13.5
Tryptophan	3.1	1.4
Valine	12.2	12.6

* The proportion of nine of the essential amino acids needed to support growth in growing rats was recalculated from {National Academy of Sciences (1978), p. 23}.
• The proportion of these essential amino acids needed to support growth in children was recalculated from {Report of a Joint FAO/WHO Ad Hoc Expert Committee on energy and protein requirements (19 74), p. 57.

However, addition of tryptophan to the low-protein diets supplemented with methionine caused no additional enhancement of the components of the inflammatory response, after exposure to the acute inflammatory response of LPS. Furthermore, it can be concluded according to the results of the present study, that either the hypothesis of Reeds *et al.* (1994) was wrong, or that when tryptophan was added in the present study there were other amino acids with a greater deficiency in supporting the acute phase response. The other possibility is that human amino acid requirements to enhance the components of the inflammatory response were not the same as in rats.

CHAPTER 7

7. The effect of graded levels of dietary casein but with identical amounts of sulphur amino acids and arginine on growth and inflammatory responsiveness in rats

7.1 Introduction

Arginine is a major and direct substrate for both urea production, to detoxify ammonia, and for nitric oxide ($NO^{\bullet}$) production. Therefore, arginine intake, could play a critical role in modulating urea synthesis, and in modulating immunity via production of $NO^{\bullet}$ (see Figure 7-1). Arginine is considered as an essential amino acid in carnivores such as cats. In omnivores, arginine is considered as conditionally essential amino acid in certain circumstances, were the de novo arginine synthesis is insufficient to satisfy the needs during growth (Hoogenraad *et al.* 1985), severe stress (e.g. sepsis, trauma) (Kirk & Barbul, 1990; Barbul, 1986), and urea cycle defects (Batshaw *et al.* 1980; Brusilow & Batshaw, 1979).

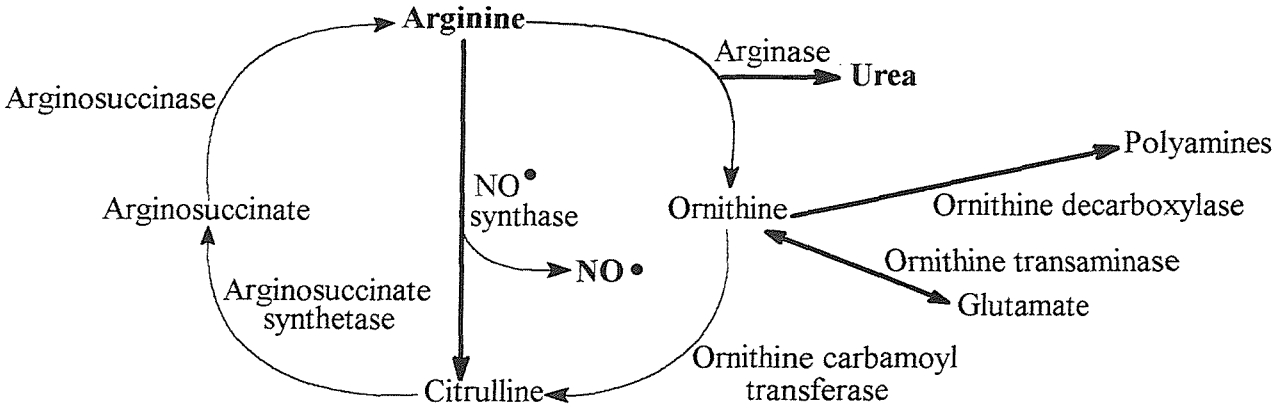
Arginine is not an essential amino acid for growth and nitrogen balance in adult rats, however in young growing rats, arginine becomes essential for optimal nitrogen balance and for growth (Rose, 1937; Borman *et al.* 1946; Milener *et al.* 1974).

Arginine is also an indirect substrate for glutamate production, and vice versa, via ornithine synthesis. Although glutamate is not an essential amino acid, and can be synthesised within the body, the requirement of glutamic acid might be increased in certain circumstances. In infected animals, where glutathione (GSH) requirements are increased, the flow of glutamate to the pathway of GSH synthesis would be increased. Hang *et al.* (1992) demonstrated that glutamine, which acts as a precursor for glutamate, was able to restore hepatic GSH concentration and protect rats against the lethal effect of acetoaminophen. Therefore, arginine supplementation might

enhance GSH concentration indirectly by providing glutamate. Furthermore, glutamine, which is synthesised from glutamate by glutamine synthetase, has been shown to be an important substrate for activated immune cells. An increased utilisation has been shown during infection (Newsholme & Parry-Billings, 1990; Souba, 1990).

Therefore, the aim of this study was to investigate the effect of arginine supplementation, in stressed and in unstressed animals fed low-protein diets, on growth, urinary end products of nitrogen and sulphur metabolism (urea-N, ammonia-N, and inorganic sulphate), and tissue GSH concentrations.

Figure 7-1 Arginine metabolism



7.2 Arginine content of the various diets

Diets were prepared by mixing dry ingredients in a mixer, this was followed by addition of maize oil. To these diets, water was added to make the diets into small biscuits that were dried in oven at 80°C for up to 48-72 hours.

In order to achieve the aim of this study and highlight on the effect of arginine supplementation to low-protein diets, rats were divided into three different dietary groups as in previous study. The amount of arginine added to all low-protein

diets used in this study was equivalent to the arginine present in the NP+M group (see Table 7.1). Furthermore, the amount of methionine was made identical in all dietary groups and was the same as in the NP+M group.

1) The first group received 180 g protein supplemented with 3 g L-methionine/kg diet. This diet contains 40.7 mM arginine, and 65 mM sulphur amino acids, and this diet was used as it a customary diet for young growing rats. This group is called the normal protein + L-methionine (NP+M) group.

2) The second group received 80 g protein supplemented with 3.9 g L-arginine, and 6.70g L-methionine/kg diet. This diet contains 40.7 mM arginine, and 65 mM sulphur amino acids. This group is called the low protein + L-methionine + L-arginine (LP+M+A) group.

3) The third group received 60 g protein supplemented with 4.7 g L-arginine, and 7.45 g L-methionine/kg diet. This diet contains 40.7 mM arginine, and 65 mM sulphur amino acids. This group is called the very low protein + L-methionine + L-arginine (VLP+M+A) group.

The low-protein diets were, however, relatively deficient in a wide range of essential amino acids as shown in Table 4.2, chapter 4.

7.3 Experimental protocol

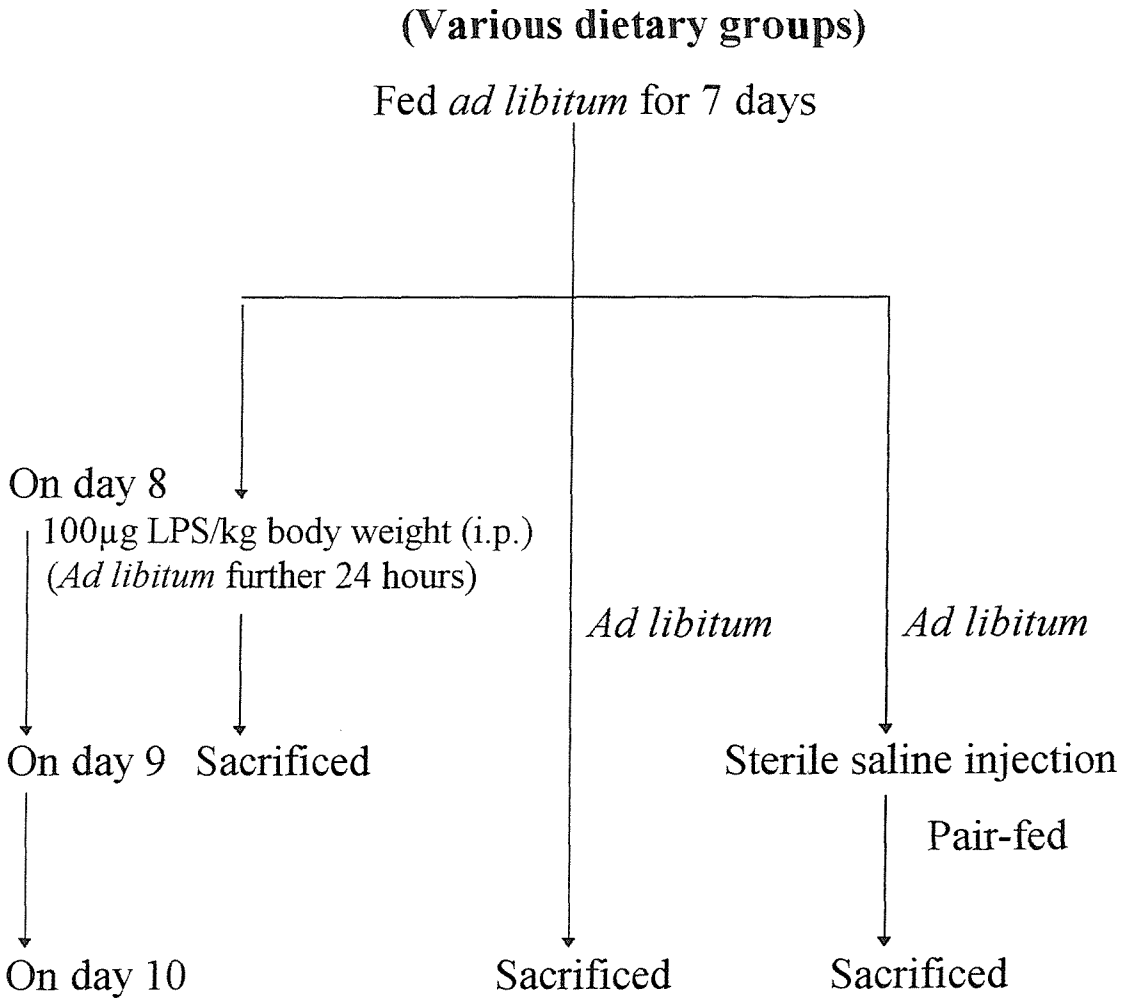
Male weanling wistar rats from Southampton University Medical School Colony weighing 50-70 g were housed individually in plastic metabolic cages and were maintained at 22 ± 1°C with a 12-12 hour light-dark cycle (Lights on at 7:30 AM).

Rats were divided into three different dietary groups (n=12 for the NP+M group, and n=18 for the LP+M+A and the VLP+M+A groups) and fed *ad libitum* the semi-synthetic diets for 7 days. On day 8, animals from each group were subdivided into three treatment groups, and fed, injected, and biopsied as described earlier in chapter 4, section 4.3 (see Figure 7-2).

Table 7.1 Diets composition (g/kg)

Component	180 g protein/kg	80 g protein/kg	60 g protein/kg
Casein	204	91	68
L-arginine	0	3.9	4.7
L-methionine	3	6.70	7.45
Cellulose	100	100	100
Sucrose	319	373	384
Maize starch	319	373	384
Maize oil	30	30	30
Vitamin mix	5	5	5
Mineral mix	20	20	20

Figure 7-2 Schematic representation of the experimental protocol



7.4 Results

7.4.1 Growth, food intake, sulphur amino acid intake, and organ weights

Average daily weight gain, food intake, and food, energy and protein efficiencies before starting treatments

Table 7.2 shows that animals fed the VLP diet supplemented with arginine and methionine had the lowest weight gain. The average daily weight gain of animals fed the LP+M+A diet was lower than animals fed the NP+M diet, but it was higher than animals fed the VLP+M+A diet.

The average daily food intake was similar between animals fed the NP+M and animals fed the LP+M+A diet, and between animals fed the LP+M+A diet and animals fed the VLP+M+A diet. However, the amount of food eaten by animals fed the VLP+M+A diet was significantly lower than animals fed the NP+M diet (Table 7.2).

There was a progressive reduction in the food efficiency (g weight gain/g food intake) and energy efficiency (mg weight gain/kJ eaten) as dietary protein content was decreased (Table 7.2). When the protein content in the diet was decreased from 180 g to 80 g/kg diet, protein efficiency (g weight gain/g protein eaten) was significantly increased. However, further reduction in protein content of the diet did not increase the protein efficiency further. The protein efficiency of the VLP+M+A group was lower than in the LP+M+A group, and higher than in the NP+M group (Table 7.2).

Comparison between the data in the present chapter and that in chapter 5 (Table 5.2) indicates that addition of arginine to the LP+M and VLP+M diets produces a significant reduction in the protein and energy efficiencies.

Table 7.2 Average daily weight gain, food intake, and food, protein and energy efficiency of rats fed diets of graded casein content but with identical amounts of sulphur amino acids and arginine, before starting treatments

Dietary groups g protein/kg diet	NP+M 180+M	LP+M+A 80+M+A	VLP+M+A 60+M+A	one-way ANOVA p-value	 F ratio
Weight gain (g)	6.2 ± 0.19[a]	4.2 ± 0.14[b]	2.6 ± 0.07[c]	p= 0.000	F=167.86
Food intake (g)	13.1 ± 0.30[a]	12.5 ± 0.35[a b]	11.7 ± 0.24[b]	p= 0.008	F=5.40
Sulphur amino acid intake (µmol)	854 ± 20[a]	815 ± 23[ab]	759 ± 16[b]	p= 0.008	F=5.40
Weight gain/food intake (g/g)	0.47 ± 0.01[a]	0.34 ± 0.01[b]	0.22 ± 0.01[c]	p=0.000	F=271.01
Weight gain/energy intake (mg/kJ)	26.2 ± 0.65[a]	18.7 ± 0.3[b]	12.2 ± 0.3[c]	p=0.000	F=271.01
weight gain/protein intake (g/g)	2.63 ± 0.06[a]	4.21 ± 0.07[b]	3.67 ± 0.09[c]	p=0.000	F=84.23

-Results are presented as mean ± standard error of the mean, n=12 for the NP+M group, and n=18 for the LP+M+A and the VLP+M+A groups. Means within each row having different letter superscripts following the number differ significantly.

The effect of endotoxin treatment on food intake and sulphur amino acid intake

The effect of LPS on food and sulphur amino acid intake can be seen in Table 7.3. In the animals fed *ad libitum*, the food (and sulphur amino acid intake) was similar between the groups. In the pair-fed animals, the food (and sulphur amino acid intake) of the VLP+M+A group was significantly lower than in the NP+M and the LP+M+A groups. In the LPS-treated animals, the food (and sulphur amino acid intake) of animals fed the LP+M+A and VLP+M+A diets was lower than in animals fed the NP+M diet, but this difference was not statistically significant.

Due to treatments, LPS-treated rats and pair-fed controls given the LP+M+A and VLP+M+A diets had a significantly lower food intake (and sulphur amino acid intake) compared with the corresponding animals fed *ad libitum*. In the NP+M group, LPS treated-rats and pair-fed controls had a lower food intake (and sulphur amino acid intake) compared with the corresponding animals fed a*d libitum*. This difference was statistically significant when comparing the pair-fed animals with the animals fed *ad libitum*.

Table 7.3 Effect of LPS on food and sulphur amino acid intake of rats fed diets of graded casein content but with identical amounts of sulphur amino acids and arginine

Dietary groups g protein/kg diet	NP+M 180+M	LP+M+A 80+M+A	VLP+M+A 60+M+A	two-way ANOVA p-value F ratio
Food intake (g)				
ad libitum	13.7 ± 0.3 [a]	14.2 ± 0.9 [a]	13.0 ± 1.3 [a]	D=0.029 F=3.87
pair-fed	7.9 ± 0.4 [a]*	7.5 ± 0.5 [a]*	5.9 ± 0.5 [b]*	T=0.000 F=42.48
LPS	9.7 ± 2.0 [a]	7.6 ± 1.0 [a]*	6.0 ± 0.5 [a]*	I=0.620 F=0.67
Sulphur amino acid intake (µmol)				
ad libitum	890 ± 18 [a]	922 ± 60 [a]	847 ± 86 [a]	D=0.029 F=3.87
pair-fed	514 ± 26 [a]*	490 ± 33 [a]*	386 ± 31 [b]*	T=0.000 F=42.48
LPS	631 ± 132 [a]	496 ± 64 [a]*	392 ± 34 [a]*	I=0.620 F=0.67

-Results are presented as mean ± standard error of the mean, n=4 per treatment group for animals fed the NP+M diet, and n=6 per treatment group for animals fed the LP+M+A and the VLP+M+A diets. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

Final body weight

The results of the final body weight can be seen in Table 7.4. In the *ad libitum,* pair-fed, and LPS-treated groups, rats fed the VLP+M+A diet had a lower body weight compared with animals fed the NP+M diet. In the pair-fed and LPS-treated animals, the final body weight of animals fed the VLP+M+A diet was also significantly lower than animals fed the LP+M+A diet. In animals fed *ad libitum*, the final body weight of the VLP+M+A group was lower than animals of the LP+M+A group, but this effect was not statistically significant.

In the VLP+M+A group, pair-fed animals and LPS-treated animals had a significantly lower body weight compared with the corresponding animals fed *ad libitum.*

Table 7.4 Final body weight of rats fed diets of graded casein content but with identical amounts of sulphur amino acids and arginine

Dietary groups g protein/kg diet	NP+M 180+M	LP+M+A 80+M+A	VLP+M+A 60+M+A	two-way ANOVA p-value	 F ratio
Body weight (g)					
ad libitum	104.7 ± 4.6^{a}	98.6 ± 5.4^{ab}	85.9 ± 3.7^{b}	D=0.000	F=42.55
pair-fed	105.9 ± 0.8^{a}	87.5 ± 3.9^{b}	75.0 ± 1.7^{c}*	T=0.005	F=6.12
LPS	102.5 ± 2.4^{a}	85.4 ± 1.5^{b}	73.3 ± 1.7^{c}*	I=0.352	F=1.14

-Results are presented as mean ± standard error of the mean, n=4 per treatment group for animals fed the NP+M diet, and n=6 per treatment group for animals fed the LP+M+A and the VLP+M+A diets. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

Organ weights

a) Total liver weight

In animals fed *ad libitum*, no difference in the total liver weight was found between the groups. In the LPS-treated and pair-fed animals, liver weight was significantly lower in the VLP+M+A group compared with the NP+M and LP+M+A groups (Table 7.5).

In the LP+M+A and VLP+M+A groups, liver weight of LPS-treated animals was significantly higher than the corresponding pair-fed animals. Pair-fed animals fed the LP+M+A and VLP+M+A diets had a lower liver weight compared with the corresponding animals fed *ad libitum*.

b) Relative liver weight

In animals fed *ad libitum*, the relative liver weight (g/kg body weight) was significantly higher in the LP+M+A and VLP+M+A groups compared with the NP+M group. In the pair-fed and in LPS-treated animals, no difference in the relative liver weight was found between the dietary groups (Table 7.5).

Due to treatments, however, in all dietary groups, the LPS-treated animals had a higher relative liver weight compared with the corresponding pair-fed controls. In the LP+M+A and VLP+M+A groups, the relative liver weight of the pair-fed animals was also lower than the corresponding animals fed *ad libitum*.

c) Total lung weight

In the *ad libitum*-fed animals, there was a progressive reduction in the total lung weight as dietary protein content was decreased. However, this reduction was not statistically significant. In the pair-fed animals, lung weight was significantly lower in the VLP+M+A groups compared to other dietary groups. In the LPS-treated animals, rats given the LP+M+A and VLP+M+A diets had a lower lung weight compared with the NP+M group (Table 7.5).

No significant difference in total lung weight was found between the groups Due to treatments.

d) Relative lung weight

Neither diet nor treatments caused a significant alteration in the relative lung weight (g/kg body weight) (Table 7.5).

Table 7.5 Organ weights of rats fed diets of graded casein content but with identical amounts of sulphur amino acids and arginine, after starting treatments

Dietary groups g protein/kg diet	NP+M 180+M	LP+M+A 80+M+A	VLP+M+A 60+M+A	two-way ANOVA p-value	 F ratio
Total liver weight (g)					
ad libitum	4.84 ± 0.1^{a}	5.52 ± 0.48^{a}	4.92 ± 0.22^{a}	D=0.002	F=7.66
pair-fed	4.20 ± 0.38^{a}	3.56 ± 0.14^{ab}*	3.04 ± 0.22^{b} *	T=0.000	F=25.72
LPS	5.26 ± 0.20^{a}	4.72 ± 0.18^{a}♠	4.00 ± 0.06^{b}♠ *	I=0.069	F=2.38
Relative liver weight (g/kg body weight)					
ad libitum	46.43 ± 1.7^{a}	55.62 ± 2.7^{b}	57.50 ± 2.3^{b}	D=0.025	F=4.04
pair-fed	39.69 ± 3.6^{a}	40.79 ± 0.5^{a}*	40.69 ± 3.4^{a}*	T=0.000	F=32.23
LPS	51.30 ± 1.9^{a} ♠	55.23 ± 1.6^{a}♠	54.61 ± 0.9^{a}♠	I=0.287	F=1.30
Total lung weight weight (mg)					
ad libitum	873 ± 68^{a}	769 ± 41^{a}	697 ± 40^{a}	D=0.000	F=17.92
pair-fed	857 ± 62^{a}	742 ± 19^{a}	650 ± 32^{b}	T=0.553	F=0.60
LPS	897 ± 22^{a}	745 ± 18^{b}	695 ± 37^{b}	I=0.962	F=0.15
Relative lung weight (g/kg body weight)					
ad libitum	8.40 ± 0.84^{a}	7.84 ± 0.30^{a}	8.10 ± 0.24^{a}	D=0.528	F=0.65
pair-fed	8.10 ± 0.64^{a}	8.57 ± 0.49^{a}	8.71 ± 0.53^{a}	T=0.079	F=2.71
LPS	8.76± 0.25^{a}	8.75 ± 0.32^{a}	9.48 ± 0.49^{a}	I=0.746	F=0.49
Total spleen weight (mg)					
ad libitum	529 ± 15^{a}	365 ± 25^{b}	278 ± 16^{c}	D=0.000	F=97.25
pair-fed	555 ± 33^{a}	338 ± 18^{b}	279 ± 28^{b}	T=0.206	F=1.65
LPS	556 ± 16^{a}	378 ± 18^{b}	322 ± 13^{c}	I=0.735	F=0.50
Relative spleen weight (g/kg body weight)					
ad libitum	5.07 ± 0.13^{a}	3.69 ± 0.11^{b}	3.24 ± 0.13^{c}	D=0.000	F=40.98
pair-fed	5.24 ± 0.32^{a}	3.86 ± 0.10^{b}	3.71 ± 0.35^{b}	T=0.000	F=10.68
LPS	5.07 ± 0.13^{a}	4.42 ± 0.19^{b}♠ *	4.40 ± 0.15^{b}*	I=0.394	F=1.05
Total thymus weight (mg)					
ad libitum	650 ± 35^{a}	576 ± 36^{ab}	473 ± 32^{b}	D=0.000	F=32.86
pair-fed	645 ± 36^{a}	501 ± 32^{b}	397 ± 8^{c}	T=0.006	F=5.78
LPS	574 ± 16^{a}	477 ± 16^{b}*	413 ± 26^{b}	I=0.566	F=0.75
Relative thymus weight (g/kg body weight)					
ad libitum	6.22 ± 0.30^{a}	5.84 ± 0.16^{a}	5.11 ± 0.32^{a}	D=0.073	F=2.80
pair-fed	6.08 ± 0.33^{a}	5.73 ± 0.22^{a}	5.31 ± 0.18^{a}	T=0.461	F=0.79
LPS	5.60 ± 0.14^{a}	5.59 ± 0.14^{a}	5.62 ± 0.26^{a}	I=0.539	F=0.79

-Results are presented as mean ± standard error of the mean, n=4 per treatment group for animals fed the NP+M diet, and n=6 per treatment group for animals fed the LP+M+A and the VLP+M+A diets. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

e) Total spleen weight

In animals fed *ad libitum*, there was a progressive reduction in the total spleen weight as dietary protein content was decreased. In the pair-fed animals, spleen weight was also decreased as the protein content in the diet was decreased. This difference was statistically significant when comparing the NP+M group with the LP+M+A and VLP+M+A groups. In the LPS-treated animals, the total spleen weight was significantly reduced as protein content in the diet was decreased (Table 7.5).

No difference in the total spleen weight was found between the groups due to treatments.

f) Relative spleen weight

In the *ad libitum*-fed animals, there was a progressive reduction in the relative spleen weight (g/kg body weight) as dietary protein content was decreased. In the pair-fed and in LPS-treated animals, the relative spleen weight was significantly lower in the LP+M+A and VLP+M+A groups when compared with the NP+M group (Table 7.5).

In the LP+M+A group and VLP+M+A groups, the LPS-treated animals had a higher relative spleen weight compared with the corresponding *ad libitum*-fed animals. In the LP+M+A group, the relative spleen weight of LPS-treated animals was also higher than the corresponding pair-fed animals.

g) Total thymus weight

In all dietary groups, there was a trend for a reduction in the total thymus weight as dietary protein content was decreased. In animals fed *ad libitum*, this trend was statistically significant when comparing the NP+M group with the VLP+M+A group. In the pair-fed animals, the total thymus weight was significantly lower in the VLP+M+A group compared with the NP+M and LP+M+A group, and it was lower in the LP+M+A group compared with the NP+M group. In the LPS-treated animals, total thymus weight was significantly lower in the LP+M+A and VLP+M+A groups compared with the NP+M group (Table 7.5).

In the LP+M+A group, the LPS-treated animals had a significantly lower thymus weight compared with the corresponding animals fed *ad libitum*.

h) Relative thymus weight

Neither diet nor treatments caused a significant alteration in the relative thymus weight (g/kg body weight) (Table 7.5).

7.4.2 Glutathione concentration

a) Liver glutathione concentration

Diets and treatments exerted a significant effect on GSH concentration (Table 7.6). In the *ad libitum* groups, rats fed the VLP+M+A diet had a significantly higher hepatic GSH concentration than other dietary groups fed the NP+M and LP+M+A diets. However, in the pair-fed animals and in LPS-treated animals, no significant changes in GSH concentration were found between dietary groups.

In all dietary groups, the LPS-treated animals had a significantly higher GSH concentration than the corresponding pair-fed animals. In all dietary groups, the pair-fed animals had a lower GSH concentration than the corresponding animals fed *ad libitum*.

b) Lung glutathione concentration

Decreasing the protein content in the diet did not alter GSH concentration in lung, while giving endotoxin exerted a significant effect on GSH concentration (Table 7.6). In all dietary groups, no significant change in lung GSH concentration was found between the groups.

Endotoxin treatment caused a significant increase in GSH concentration compared with the pair-fed controls.

c) Spleen glutathione concentration

Although animals fed the low-protein diets were able to maintain GSH concentration in liver and lung in all the three treatment groups, when compared to the corresponding animals fed the NP+M diet, they were unable to do so in spleen (Table 7.6).

In animals fed *ad libitum*, and in pair-fed animals, the low-protein dietary groups (the LP+M+A and VLP+M+A groups) had a significantly lower GSH concentration than the NP+M group. In the LPS-treated animals, spleen GSH concentration was lower in the VLP+M+A group compared with other dietary groups fed the NP+M and LP+M+A diets. Restriction in food intake and endotoxin-treatment did not exert a significant effect on spleen GSH concentration, unlike the situation in liver and lung.

d) Thymus glutathione concentration

LPS-treated animals fed the VLP+M+A diet were unable to maintain thymus GSH concentration to the same level found in LPS-treated animals fed the NP+M diet (Table 7.6).

Neither food restriction nor LPS treatment caused a significant change in thymus GSH concentration compared with the unstressed animals.

Table 7.6 Glutathione concentration in liver, lung, spleen, and thymus of rats fed diets of graded casein content but with identical amounts of sulphur amino acids and arginine, after starting treatments (μmol/g tissue)

Dietary groups g protein/kg diet	NP+M 180+M	LP+M+A 80+M+A	VLP+M+A 60+M+A	two-way ANOVA p-value	 F ratio
Liver					
ad libitum	6.10 ± 0.14 [a]	6.48 ± 0.56 [a]	8.05 ± 0.36[b]	D=0.048	F=3.29
pair-fed	2.47 ± 0.35 [a]*	2.64 ± 0.43 [a]*	2.83 ± 0.59 [a]*	T=0.000	F=103.51
LPS	6.81 ± 0.47 [a]♠	7.27 ± 0.18 [a]♠	7.18 ± 0.24 [a]♠	I=0.183	F=1.64
Lung					
ad libitum	1.90 ± 0.03 [a]	1.88 ± 0.08 [a]	1.94 ± 0.10 [a]	D=0.652	F=0.43
pair-fed	1.66 ± 0.05 [a]*	1.70 ± 0.09 [a]	1.59 ± 0.12 [a]	T=0.000	F=11.86
LPS	1.98 ± 0.04 [a]♠	2.06 ± 0.04 [a]♠	1.92 ± 0.08 [a]♠	I=0.748	F=0.48
Spleen					
ad libitum	3.21 ± 0.10 [a]	2.83 ± 0.12[b]	2.56 ± 0.11[b]	D=0.000	F=16.00
pair-fed	3.19 ± 0.07 [a]	2.85 ± 0.08[b]	2.67 ± 0.17[b]	T=0.261	F=1.39
LPS	3.17 ± 0.11 [a]	3.04 ± 0.05 [a]	2.82 ± 0.07[b]	I=0.694	F=0.56
Thymus					
ad libitum	2.07 ± 0.43 [a]	2.02 ± 0.05 [a]	1.94 ± 0.03 [a]	D=0.000	F=10.24
pair-fed	2.07 ± 0.07 [a]	2.03 ± 0.04 [a]	1.90 ± 0.03 [a]	T=0.078	F=2.73
LPS	2.17 ± 0.05 [a]	2.07 ± 0.04 [ab]	1.98 ± 0.05[b]	I=0.917	F=0.23

-Results are presented as mean ± standard error of the mean, n=4 per treatment group for animals fed the NP+M diet, and n=6 per treatment group for animals fed the LP+M+A and the VLP+M+A diets. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

7.4.3 Protein in liver and lung

a) Liver protein concentration

In animals fed *ad libitum*, the protein concentration in liver was significantly lower in the LP+M+A and VLP+M+A groups than in the NP+M group. In the pair-fed animals, no difference in liver protein concentration was found between the groups. In the LPS-treated animals, the VLP+M+A group had a lower protein concentration than in the NP+M group (Table 7.7).

In both the low-protein groups, pair-fed animals had a higher protein concentration than the corresponding *ad libitum* and LPS-treated animals. However, in the LP+M+A group, this difference was not statistically significant.

b) Total liver protein

In all dietary groups, there was a trend for a reduction in the total liver protein when the dietary protein content was decreased to 60 g/kg diet. This was statistically significant in the pair-fed and LPS-treated animals (Table 7.7).

In the VLP+M+A group, animals fed *ad libitum* had a significantly higher total liver protein than the corresponding pair-fed and LPS-treated animals. In all dietary groups, the LPS-treated animals had a significantly more liver protein than the corresponding pair-fed controls.

c) Total liver protein/kg body weight

In all dietary groups, no significant difference was found in the amount of liver protein, when it was expressed relative to body weight (Table 7.7).

In all dietary groups, the LPS-treated animals had a significantly increased the relative liver protein values compared with the corresponding pair-fed controls. Pair-fed controls given the LP+M+A and VLP+M+A diets had a lower relative liver protein compared with the corresponding animals fed *ad libitum*.

Table 7.7 Protein in liver and lung of rats fed diets of graded casein content but with identical amounts of sulphur amino acids and arginine, after starting treatments

Dietary groups g protein/kg diet	NP+M 180+M	LP+M+A 80+M+A	VLP+M+A 60+M+A	two-way ANOVA p-value F ratio
Protein in liver (mg/g liver)				
ad libitum	148.4 ± 1.3 [a]	132.6 ± 4.0 [b]	131.3 ± 4.4 [b]	D=0.017 F=4.53
pair-fed	157.4 ± 5.1 [a]	157.5 ± 3.4 [a]*	155.0 ± 5.3 [a]*	T=0.000 F=12.31
LPS	154.4 ± 4.5 [a]	144.2 ± 5.9 [ab]	138.1 ± 4.6 [b]♠	I=0.458 F 0.93
Total liver protein (mg/ total liver)				
ad libitum	718 ± 16 [a]	746 ± 61 [a]	646 ± 35 [a]	D=0.000 F=18.38
pair-fed	657 ± 41 [a]	560 ± 21 [a]	468 ± 30 [b]*	T=0.000 F=14.06
LPS	812 ± 39 [a]♠	676 ± 15 [b]♠	551 ± 17 [c]♠*	I=0.115 F=1.99
Relative liver protein (g/kg body weight)				
ad libitum	6.89 ± 0.24 [a]	7.53 ± 0.37 [a]	7.51 ± 0.16 [a]	D=0.461 F=0.79
pair-fed	6.20 ± 0.39 [a]	6.42 ± 0.11 [a]*	6.26 ± 0.44 [a]*	T=0.000 F=20.67
LPS	7.91 ± 0.25 [a]♠*	7.93 ± 0.22 [a]♠	7.52 ± 0.18 [a]♠	I=0.591 F=0.71
Lung protein (mg/g lung)				
ad libitum	78.3 ± 2.5 [a]	81.8 ± 4.2 [a]	83.4 ± 2.4 [a]	D=0.575 F=0.56
pair-fed	83.8 ± 2.1 [a]	78.3 ± 1.9 [a]	82.6 ± 3.0 [a]	T=0.658 F=0.42
LPS	82.4 ± 3.5 [a]	83.4 ± 2.7 [a]	84.0 ± 1.8 [a]	I=0.631 F=0.65
Total lung protein (mg/ total lung)				
ad libitum	69.7 ± 7.2 [a]	62.2 ± 1.8 [a]	58.1 ± 3.7 [a]	D=0.000 F=12.13
pair-fed	73.9 ± 6.7 [a]	58.1 ± 2.4 [a]	53.7 ± 3.2 [a]	T=0.640 F=0.45
LPS	74.1 ± 4.9 [a]	62.1 ± 2.2 [ab]	58.4 ± 3.6 [b]	I=0.837 F=0.36
Relative lung protein (mg/kg body weight)				
ad libitum	0.67 ± 0.09 [a]	0.64 ± 0.04 [a]	0.68 ± 0.04 [a]	D=0.439 F=0.84
pair-fed	0.70 ± 0.07 [a]	0.67 ± 0.05 [a]	0.72 ± 0.06 [a]	T=0.133 F=2.12
LPS	0.72 ± 0.04 [a]	0.73 ± 0.04 [a]	0.80 ± 0.04 [a]	I=0.977 F=0.11

-Results are presented as mean ± standard error of the mean, n=4 per treatment group for animals fed the NP+M diet, and n=6 per treatment group for animals fed the LP+M+A and the VLP+M+A diets. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

d) Lung protein concentration

Neither diet nor treatments caused a significant alteration in the lung protein concentration (Table 7.7).

e) Total lung protein

There was a trend for a reduction in the total lung protein as dietary protein content was decreased (Table 7.7). This difference was statistically significant in LPS-treated animals, when comparing the NP+M group with the VLP+M+A group.

Due to treatments, no significant difference in total lung protein was found in any dietary group.

f) Total lung protein/kg body weight

All animals were able to maintain the relative lung protein (Table 7.7). Changes in the protein content in the diet, endotoxin treatment, and restriction in food intake, were unable to significantly change the relative lung protein.

7.4.4 Inorganic sulphate excretion and urinary nitrogenous end products of amino acids metabolism: Urea-N and ammonia-N excretion

Inorganic sulphate excretion, and inorganic sulphate excretion as a percentage of sulphur amino acid intake-average of two days before starting treatments

The inorganic sulphate excretion in all dietary groups was similar (Table 7.8). However, when the results of SO_4 excretion were expressed as a percentage of sulphur amino acid intake, it can be seen from Table 7.8, that there was a trend for an increase in the ratio as dietary protein content was decreased. The increase was statistically significant when comparing the VLP+M+A group with the NP+M and LP+M groups.

The ratio of SO_4 excretion to sulphur amino acid intake, in the present study, was greater than the ratio when methionine alone was added to the low-protein diets

(chapter 5, Table 5.11). The ratio increased from 37% in the LP+M dietary group to 39% in the LP+M+A dietary group. This increase in the ratio was not statistically significant. In the very low-protein dietary groups, the ratio increased from 40% after addition of methionine to 46% after addition of methionine and arginine. This increase in the ratio was statistically significant.

Table 7.8 Inorganic sulphate excretion, and the percentage of sulphur amino acid intake excreted as inorganic sulphate of rats fed diets of graded casein content but with identical amounts of sulphur amino acids and arginine-average of two days before starting treatments (µmol/day)

Dietary groups g protein/kg diet	NP+M 180+M	LP+M+A 80+M+A	VLP+M+A 60+M+A	one-way ANOVA p-value	 F ratio
SO_4 excretion	329 ± 15.9 [a]	318 ± 9.5 [a]	345 ± 11.1 [a]	p= 0.277	F=1.34
(SO_4 excretion/sulphur amino acid intake) x 100	36.2 ± 1.5 [a]	39.3 ± 1.2 [a]	45.8 ± 1.1 [b]	p=0.000	F=15.45

-Results are presented as mean ± standard error of the mean, n=12 for the NP+M, LP+M+A and the VLP+M+A groups. Means within each row having different letter superscripts following the number differ significantly.

Inorganic sulphate excretion after starting treatments

The amount of SO_4 excreted in urine was similar between all dietary groups (Table 7.9). In the VLP+M+A group, food restriction and exposure to the inflammatory response of LPS caused a significant reduction in SO_4 excretion compared to values in animals fed *ad libitum*. In other dietary groups, there was a trend for a reduction in SO_4 excretion after food restriction and LPS-treatment, in which the magnitude of the reduction was greater in the LP+M+A group than in the NP+M group, but this difference was not statistically significant.

Table 7.9 Inorganic sulphate excretion of rats fed diets of graded casein content but with identical amounts of sulphur amino acids and arginine, after starting treatments (μmol/day)

Dietary groups	NP+M	LP+M+A	VLP+M+A	two-way ANOVA	
g protein/kg diet	180+M	80+M+A	60+M+A	p-value	F ratio
ad libitum	264 ± 29 [a]	307 ± 25 [a]	322 ± 17 [a]	D=0.488	F=0.74
pair-fed	233 ± 10 [a]	257 ± 22 [a]	223 ± 19 [a] *	T= 0.001	F= 9.68
LPS	228 ± 17 [a]	219 ± 27 [a]	200 ± 18 [a] *	I=0.539	F=0.79

-Results are presented as mean ± standard error of the mean, n=4 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

Ammonia-N and urea-N excretion-average of two days before starting treatments

The LP+M+A and VLP+M+A groups had a significantly lower ammonia-N and urea-N excretion compared with the NP+M group (Table 7.10). No significant difference was found in ammonia-N and urea-N excretion between the LP+M+A and VLP+M+A groups.

Table 7.10 Ammonia-N and urea-N excretion of rats fed diets of graded casein content but with identical amounts of sulphur amino acids and arginine-average of two days before starting treatments (μmol/day)

Dietary groups	NP+M	LP+M+A	VLP+M+A	one-way ANOVA	
g protein/kg diet	180+M	80+M+A	60+M+A	p-value	F ratio
Ammonia-N	813± 34 [a]	375 ± 12 [b]	349± 10 [b]	p=0.000	F=143.00
Urea-N	10319 ± 470[a]	1809 ± 70 [b]	1862 ± 100 [b]	p=0.000	F=279.46

-Results are presented as mean ± standard error of the mean, n=12 per group. Means within each row having different letter superscripts following the number differ significantly.

Ammonia-N and urea-N excretion after starting treatments

a) Ammonia-N excretion after starting treatments

In all dietary groups, ammonia-N excretion was lower in animals fed the low-protein diets than in animals fed the NP+M diet (Table 7.11).

In all dietary groups, there was a trend for a reduction in ammonia-N excretion after giving endotoxin compared with values from *ad libitum*-fed animals. The reduction was statistically significant in the NP+M and VLP+M+A groups.

b) Urea-N excretion after starting treatments

Animals fed the low-protein diets exhibited lower amounts of urea-N in urine than animals fed the NP+M diet (Table 7.11). Exposure to the inflammatory response of LPS caused a slight increase in urea-N excretion compared with pair-fed animals, but this increase was not statistically significant. Pair-fed animals given the VLP+M+A diet had a significantly lower urea-N excretion when compared with the corresponding animals fed *ad libitum*.

While NH_3 excretion was not changed by addition of arginine to the low-protein diets supplemented with methionine, there were significant increases in urea-N excretion. This effect was apparent in all treatment groups (see Table 5.14, chapter 5 & Table 7.11).

Table 7.11 Ammonia-N and urea-N excretion of rats fed diets of graded casein content but with identical amounts of sulphur amino acids and arginine, after starting treatments (μmol/day)

Dietary groups g protein/kg diet	NP+M 180+M	LP+M+A 80+M+A	VLP+M+A 60+M+A	two-way ANOVA p-value	 F ratio
Ammonia-N					
ad libitum	741 ± 32^{a}	341 ± 31^{b}	329 ± 29^{b}	D=0.000	F= 54.10
pair-fed	511 ± 38^{a}*	360 ± 39^{b}	275 ± 20^{b}	T=0.000	F=13.23
LPS	457 ± 56^{a}*	255 ± 21^{b}	215 ± 23^{b}*	I=0.009	F=4.23
Urea-N					
ad libitum	10051 ± 697^{a}	1793 ± 76^{b}	1834 ± 132^{b}	D=0.000	F=445.11
pair-fed	8283 ± 425^{a}	1631 ± 253^{b}	1235 ± 158^{b} *	T=0.017	F=4.78
LPS	8520 ± 477^{a}	1816 ± 78^{b}	1515 ± 129^{b}	I=0.142	F=1.89

-Results are presented as mean ± standard error of the mean, n=4 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

7.4.5 Relationship between parameters measured in the study

The ratio of glutathione to protein after starting treatments

In the unstressed animals fed *ad libitum*, the results suggest that rats fed the VLP+M+A diet had more substrates (glycine, glutamate, and cysteine) directed toward hepatic GSH synthesis than towards hepatic protein compared with other dietary groups (Table 7.12). In other words, in animals fed *ad libitum*, the ratio of hepatic GSH to hepatic protein was significantly higher in the VLP+M+A group compared with other dietary groups (the NP+M and LP+M+A groups). In all dietary groups, food restriction caused a dramatic reduction in the ratio. However, all dietary groups were able to increase the ratio of GSH to protein, after giving endotoxin, compared with pair-fed controls. In the pair-fed animals, no significant difference in the ratio was found between dietary groups. In the LPS-treated animals, the ratio was slightly increased in the LP+M+A and VLP+M+A groups compared with the NP+M groups, but this difference was not statistically significant. In the VLP+M+A group, the ratio of hepatic GSH to hepatic protein was lower in the endotoxin-treated animals compared with the corresponding animals fed *ad libitum*.

Table 7.12 The ratio of hepatic glutathione to hepatic protein and to inorganic sulphate excretion of rats fed graded casein content but with identical amounts of sulphur amino acids and arginine, after starting treatments

Dietary groups g protein/kg diet	NP+M 180+M	LP+M+A 80+M+A	VLP+M+A 60+M+A	two-way ANOVA p-value	 F ratio
GSH/ protein (nmol/mg)					
ad libitum	41.1 ± 1.3 [a]	49.0 ± 4.2 [a]	61.4 ± 2.0 [b]	D=0.002	F=7.28
pair-fed	15.7 ± 2.3 [a]*	16.7 ± 2.6 [a]*	18.4 ± 4.0 [a]*	T=0.000	F=106.5
LPS	44.4 ± 4.0 [a]♠	51.0 ± 3.4 [a]♠	52.1 ± 1.8 [a]♠*	I=0.085	F=2.22
GSH/SO_4 (nmol per liver /µmol per day)					
ad libitum	119 ± 7 [a]	116 ± 24 [a]	125 ± 6 [a]	D=0.819	F=0.20
pair-fed	44 ± 6 [a]*	32 ± 6 [a]*	30 ± 3 [a]*	T= 0.000	F=62.19
LPS	159 ± 16 [a]♠	174 ± 20 [a]♠	148 ± 20 [a]♠	I=0.700	F=0.55

-Results are presented as mean ± standard error of the mean. For the ratio of hepatic glutathione to hepatic protein, n=4 per treatment group for animals fed the NP+M diet, and n=6 per treatment group for animals fed the LP+M+A and the VLP+M+A diets. For the ratio of hepatic glutathione to inorganic sulphate excretion, n=4 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

The ratio of total hepatic glutathione to inorganic sulphate excretion in urine after starting treatment

No significant difference in the ratio of hepatic GSH to SO_4 excretion was found between dietary groups (Table 7.12).

Animals given endotoxin dramatically increased the ratio of GSH to SO_4 excretion compared with the pair-fed controls. Pair-fed animals had a significantly lower ratio than animals fed *ad libitum*.

The ratio of ammonia-N and urea-N excretion to nitrogen intake-average of two days before starting treatments

a) The ratio of ammonia-N to nitrogen intake

More ammonia-N was excreted in urine/mg of nitrogen eaten when dietary protein content in the diet was decreased to 60 g/kg diet (Table 7.13). However, no difference in the ratio was found between the NP+M group and the LP+M+A group.

Addition of arginine to the low-protein diets supplemented with methionine had no effect upon the relationship between urinary ammonia-N and nitrogen intake.

b) The ratio of urea-N to nitrogen intake

The ratio of urea-N to nitrogen intake was significantly lower in animals fed the low-protein diets compared with animals fed the NP+M diet (Table 7.13). Reducing the amount of protein in the diet from 80 g to 60 g/kg diet, while maintaining a constant arginine and methionine intake, caused a significant increase in the ratio of urea-N to nitrogen intake.

Unlike the ratio of ammonia-N to nitrogen intake that of urea-N excretion to nitrogen intake increased, not surprisingly. While in chapter 5 it can be seen that the severity of dietary protein deficiency does not influence the ratio. The ratio for the 80 g and 60 g/kg diets supplemented with methionine was almost identical (see Table 5.16). However, in Table 7.13, it can be seen that addition of arginine led to an increase in the ratio, indicating a worsening in the efficiency of protein utilisation for growth. There is further evidence for this effect by examining the growth efficiencies of the animals fed the low-protein diets in Table 7.2.

Table 7.13 The ratio of ammonia-N and urea-N excretion to nitrogen intake of rats fed diets of graded casein content but with identical amounts of sulphur amino acids and arginine-average of two days before starting treatments (mg/mg)

Dietary groups	NP+M	LP+M+A	VLP+M+A	one-way ANOVA	
g protein/kg diet	180+M	80+M+A	60+M+A	p-value	F ratio
NH_3-N/N intake	0.029 ± 0.001 [a]	0.029 ± 0.001 [a]	0.036 ± 0.0.001 [b]	p=0.000	F=19.76
Urea-N/N intake	0.350 ± 0.011 [a]	0.132 ± 0.004 [b]	0.189 ± 0.007 [c]	p=0.000	F=194.97

-Results are presented as mean ± standard error of the mean, n=12 per group. Means within each row having different letter superscripts following the number differ significantly.

The ratio of ammonia-N and urea-N excretion to nitrogen intake after starting treatments

a) The ratio of ammonia-N to nitrogen intake

In all dietary groups, there was an increase in the ratio of ammonia-N to nitrogen intake as dietary protein content was decreased to 60 g/kg diet (Table 7.14).

In the animals fed *ad libitum*, the VLP+M+A group had a significantly higher ratio than the NP+M+A group. In the pair-fed animals, a significant increase in the ratio was observed in animal fed the VLP+M+A diet compared with other dietary groups fed the NP+M and LP+M+A diets. In the LPS-treated animals, there was a trend for an increase in the ratio as dietary protein content in the diet was decreased. This difference was not statistically significant.

In all dietary groups, the ratio was slightly decreased after giving endotoxin compared with pair-fed animals, but this difference was not statistically significant. Pair-fed animals given the LP+M+A and VLP+M+A diets had a higher ratio than the corresponding animals fed *ad libitum*.

b) The ratio of urea-N to nitrogen intake

In the animals fed *ad libitum*, the ratio of urea-N to nitrogen intake was higher in the NP+M group compared with the low-protein dietary groups (Table 7.14). Animals fed the VLP+M+A diet had a significantly higher ratio than animals

fed the LP+M+A diet. In the pair-fed animals, the ratio was also higher in the NP+M group compared to other dietary groups. The VLP+M+A group had a higher ratio than the LP+M+A group, but this difference was not statistically significant. In the LPS-treated animals, the NP+M group had a higher ratio than the LP+M+A and VLP+M+A groups. This difference was statistically significant when comparing the NP+M group with the LP+M+A group. LPS-treated animals fed the VLP+M+A diet had a higher ratio than animals fed the LP+M+A diet.

In all dietary groups, there was a trend for a higher ratio in the pair-fed animals when compared with values from animals fed *ad libitum*, but this difference was statistically significant in the NP+M group. Animals treated with LPS excreted more urea-N in urine/mg nitrogen eaten compared with animals fed *ad libitum*. This difference was statistically significant in the VLP+M+A group.

Table 7.14 The ratio of ammonia-N and urea-N excretion to nitrogen intake of rats fed graded casein content but with identical amounts of sulphur amino acids and arginine, after starting treatments (mg/mg)

Dietary groups	NP+M	LP+M+A	VLP+M+A	two-way ANOVA	
g protein/kg diet	180+M	80+M+A	60+M+A	p-value	F ratio
NH_3-N/N intake					
ad libitum	0.026 ± 0.008 [a]	0.025 ± 0.003 [ab]	0.034 ± 0.002 [b]	D=0.000	F=25.29
pair-fed	0.031 ± 0.003 [a]	0.043 ± 0.005 [a]*	0.059 ± 0.003 [b]*	T=0.000	F=16.79
LPS	0.024 ± 0.001 [a]	0.033 ± 0.005 [a]	0.048 ± 0.007 [a]	I=0.074	F=2.42
Urea-N/N intake					
ad libitum	0.35 ± 0.02 [a]	0.13 ± 0.01 [b]	0.19 ± 0.01 [c]	D=0.000	F=62.38
pair-fed	0.51 ± 0.04 [a]*	0.20 ± 0.03 [b]	0.26 ± 0.03 [b]	T=0.000	F=11.25
LPS	0.46 ± 0.05 [a]	0.19 ± 0.02 [b]	0.33 ± 0.03 [a]*	I=0.262	F=1.40

-Results are presented as mean ± standard error of the mean, n=4 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

The ratio of inorganic sulphate excretion to ammonia-N and urea-N excretion-average of two days before starting treatments

a) The ratio of inorganic sulphate excretion to ammonia-N excretion

Animals fed the NP+M diet had a dramatically lower ratio (SO_4/NH_3-N ratio) compared with animals fed the low-protein diets (Table 7.15). The ratio was slightly increased in the VLP+M+A group compared with the LP+M+A group. The difference was statistically significant.

Table 7.15 The ratio of inorganic sulphate excretion to ammonia-N and urea-N excretion of rats fed graded casein content but with identical amounts of sulphur amino acids and arginine-average of two days before starting treatments (μmol/μmol)

Dietary groups	NP+M	LP+M+A	VLP+M+A	one-way ANOVA	
g protein/kg diet	180+M	80+M+A	60+M+A	p-value	F ratio
SO_4/NH_3-N	0.40 ± 0.01[a]	0.84 ±0.02[b]	0.99 ± 0.02[c]	p=0.000	F=333.22
SO_4/Urea-N	0.031 ± 0.001[a]	0.182 ±0.005[b]	0.189 ± 0.007[b]	p=0.000	F=297.37

-Results are presented as mean ± standard error of the mean, n=12 per group. Means within each row having different letter superscripts following the number differ significantly.

b) The ratio of inorganic sulphate excretion to urea-N excretion

The ratio of SO_4/urea-N excretion was clearly lower in the NP+M group compared with the low-protein dietary groups (Table 7.15). No difference in the ratio was found between the LP+M+A group and the VLP+M+A group.

The ratio of inorganic sulphate excretion to ammonia-N and urea-N excretion after starting treatments

a) The ratio of inorganic sulphate excretion to ammonia-N excretion

In all dietary groups, the ratio of SO_4/NH_3-N excretion was significantly higher in the low-protein dietary groups compared with the NP+M group (Table

7.16). No difference in the ratio was found between the LP+M+A group and the VLP+M+A group.

No significant difference in the ratio was found due to the effect of treatments.

Table 7.16 The ratio of inorganic sulphate excretion to ammonia-N and urea-N excretion of rats fed graded casein content but with identical amounts of sulphur amino acids and arginine, after starting treatments (μmol/μmol)

Dietary groups g protein/kg diet	NP+M 180+M	LP+M+A 80+M+A	VLP+M+A 60+M+A	two-way ANOVA p-value	 F ratio
SO_4/NH_3-N					
ad libitum	0.36 ± 0.03 [a]	0.91 ± 0.07 [b]	0.93 ± 0.02 [b]	D=0.000	F=60.79
pair-fed	0.46 ± 0.02 [a]	0.73 ± 0.07 [b]	0.81 ± 0.03 [b]	T=0.118	F=2.32
LPS	0.51 ± 0.04 [a]	0.86 ± 0.08 [b]	0.90 ± 0.08 [b]	I=0.092	F=2.24
SO_4/Urea-N					
ad libitum	0.026 ± 0.002 [a]	0.172 ± 0.015 [b]	0.166 ± 0.003 [b]	D=0.000	F=166.0
pair-fed	0.028 ± 0.002 [a]	0.165 ± 0.018 [b]	0.187 ± 0.016 [b]	T=0.005	F=6.53
LPS	0.027 ± 0.002 [a]	0.135 ± 0.008 [b]	0.132 ± 0.006 [b]♠*	I=0.107	F=2.12

-Results are presented as mean ± standard error of the mean, n=4 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means within each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

b) The ratio of inorganic sulphate excretion to urea-N excretion

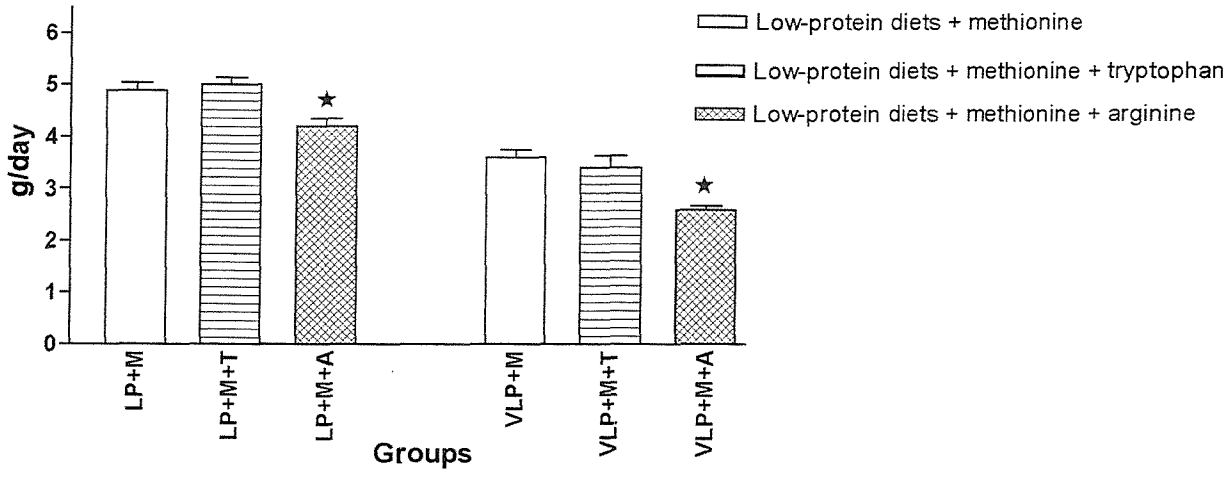
In all dietary groups, the ratio of SO_4 excretion to urea-N excretion was significantly lower in the NP+M group compared with the other low-protein dietary groups (Table 7.16). No difference in the ratio was found between the LP+M+A and the VLP+M+A groups.

LPS-treated animals fed the VLP+M+A diet had a significantly lower ratio than the corresponding animals fed *ad libitum* and pair-fed controls.

7.5 Discussion

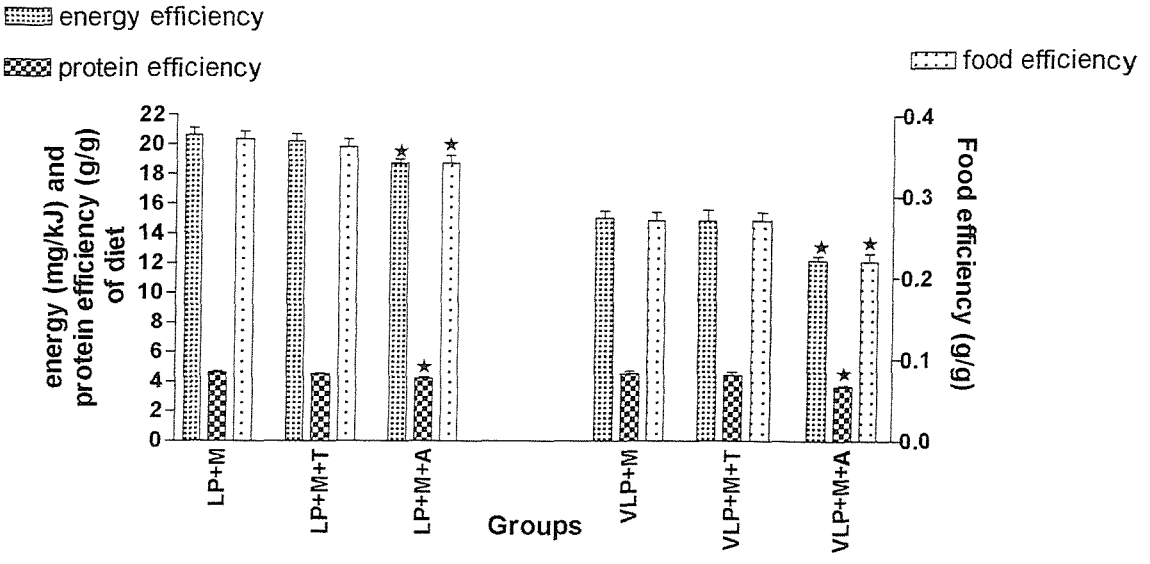
It has been recognised, for a long time, that arginine is an important amino acid for growth in the rat and may be important for optimal immune functions in man and other species. However, the average daily weight gain of animals fed the low-protein diets supplemented with methionine did not improve after addition of arginine. Indeed, the weight gain of animals fed the low-protein diets supplemented with methionine and arginine was significantly lower than animals fed the same levels of protein supplemented with methionine alone (CHAPTER 5), or supplemented with methionine and tryptophan (CHAPTER 6) see Figure 7-3. The food efficiency (g weight gain/g food eaten) and energy efficiency (mg weight gain /kJ energy intake) of animals fed these diets were also significantly lower than in animals fed the same levels of protein, but supplemented with methionine alone (CHAPTER 5), or with methionine and tryptophan (CHAPTER 6), see Figure 7-4. Furthermore, the protein efficiency (g weight gain/g protein eaten) was also significantly reduced in animals fed the LP+M+A and VLP+M+A diets compared with animals fed the same levels of protein, but with only methionine or with methionine and tryptophan supplementation (Figure 7-4).

Figure 7-3 Average daily weight gain of rats fed low-protein diets supplemented with methionine, methionine and tryptophan, or methionine and arginine



- Results are means ± SE, n=18.
⋆ Significantly different from animals fed the same level of protein, but without arginine supplementation.

Figure 7-4 Energy, protein, and food efficiency of rats fed low-protein diets supplemented with methionine, methionine and tryptophan, or methionine and arginine



Results are means ± SE, n=18.

★ Significantly different from animals fed the same level of protein, but without arginine supplementation

It can be concluded that addition of arginine to the low-protein diets was not an effective way for optimising growth, despite is role as a semi-essential amino acid for growing rats as has been reported in some studies (Rose, 1937; Borman *et al.* 1946; Milener *et al.* 1974). The poor utilisation of arginine for anabolic pathways could be the result of ingestion of misappropriate amount of other essential amino acids by animals fed the low-protein diets. One way of relieving the stress caused by the addition of arginine, causing overload of nitrogen, is by reducing food intake. This phenomenon occurred in animals fed the low-protein diets. For example, in animals fed the 60 g protein/kg diet, the daily food intake was reduced from 13.4 g and 12.6 g after addition of methionine and methionine and tryptophan respectively to 11.7 g after addition of arginine and methionine.

Thus in unstressed animals, arginine supplementation would appear to present the animal with a metabolic stress which it tried to overcome by a reduction in food

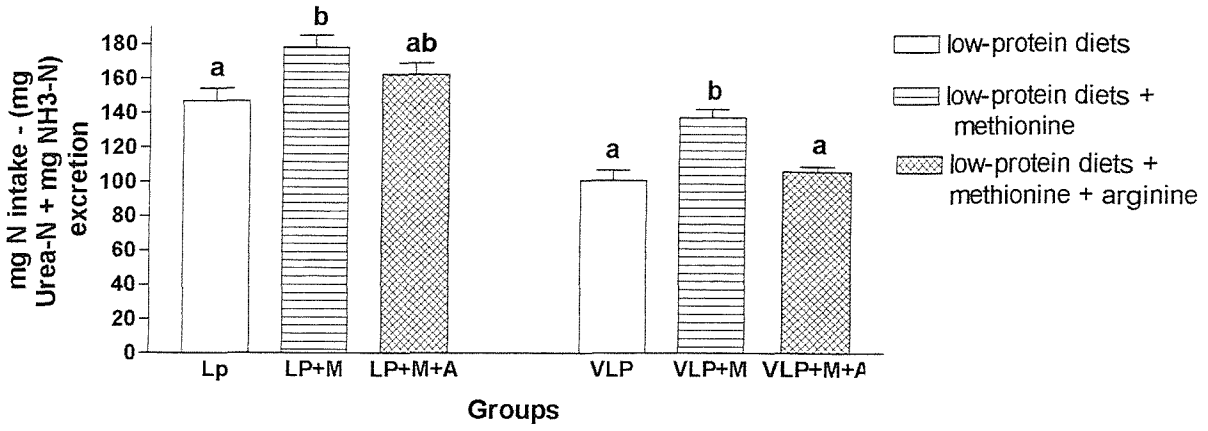
intake. However, studies in both humans and rats give a different picture of the effects of arginine under conditions of inflammatory stress.

Thirty gram of arginine-HCL supplementation, given orally to healthy subjects or intravenously to patients undergoing major surgery, significantly increased the peripheral blood lymphocyte response to mitogenic stimulus (Barbul *et al.* 1981; Barbul, 1990). A pharmacological dose of 25g-30g of arginine-HCL, administered to patients undergoing surgery, resulted in improve nitrogen balance (Elsair *et al.* 1978; Daly *et al.* 1988). Sitren & Fisher (1977) have shown no significant difference in nitrogen retention (nitrogen intake minus urea-N excretion) between mature-unstressed rats fed a low-protein diet (100 g casein/kg diet) and animals fed the same level of protein supplemented with 12 g L-arginine-HCL and 5g glycine/kg diet. When these animals were subjected to the trauma of bone fracture, nitrogen retention was still similar between the arginine supplemented and arginine-unsupplemented groups. However, in animals fed the normal-protein diet (200 g casein/kg diet) addition of arginine (24 g/kg diet), and glycine (10 g/kg diet) significantly increased nitrogen retention compared with the animals fed the same diet, but without arginine and glycine supplementation. The diets with no arginine supplementation were made isonitrogenous to their corresponding diets by adding aspartic acid. Thus traumatised rats fed the normal-protein diet benefit from arginine and glycine supplementation, but not in rats fed the low-protein diet. The same laboratory showed that the improvement in nitrogen retention of traumatised rats fed a normal-protein diet was mainly due to arginine but not glycine supplementation (Chyun & Griminger, 1984). Sitren & Fisher (1977) postulated that the improvement in nitrogen retention in traumatised rats fed the normal-protein diet, but not in animals fed the low-protein diet was due to the differences in the requirement for arginine between animals fed these diets. Thus in addition to the increase demand of arginine after injury for repairing injured tissue (e.g. collagen synthesis), and for increase urea-cycle activity, that would be expected after injury for detoxifying ammonia, animals fed the 200 g casein diet would have a higher demand of arginine as an intermediate of the urea-cycle. The authors suggests that arginine

supplementation is useful in animals fed the normal-protein diet because this diet has a high amino acid and nitrogen content. The nitrogen content of the 200 g casein diet is higher than the nitrogen requirement of adult rat (National Research Council, 1971). If the increase demand of arginine after injury in animals fed the 200 g casein diet could not be satisfied by endogenous sources and by the amount of arginine present in the 200 g casein/kg diet it becomes a limiting amino acid for the urea-cycle. Arginine may thus be diverted from protein synthesis toward the urea-cycle. As a result of this change in metabolism, protein synthesis would be diminished with the result that more amino acids need to be detoxified.

In our study, before starting treatments, no difference in nitrogen retention (nitrogen intake minus Urea-N + NH_3-N excretion) was found between animals fed the low-protein diets, without amino acid supplementation (CHAPTER 4), and animals fed the low-protein diets, supplemented with arginine and methionine (see Figure 7-5). A similarity in nitrogen retention was observed, despite the fact that nitrogen content of the diets supplemented with arginine and methionine was higher than the corresponding low-protein diets without amino acids supplementation. The LP+M+A and the VLP+M+A diets had 1900 and 2200 mg N/kg diet of extra N respectively compared with the corresponding low-protein diets, without amino acid supplementation. However, addition of methionine to the low-protein diets resulted in greater nitrogen retention than in the corresponding low-protein dietary groups with or without arginine and methionine supplementation.

Figure 7-5 Apparent nitrogen retention of rats fed either low-protein diets, low-protein diets supplemented with methionine, or low-protein diets supplemented with methionine and arginine-average of two days before starting treatments

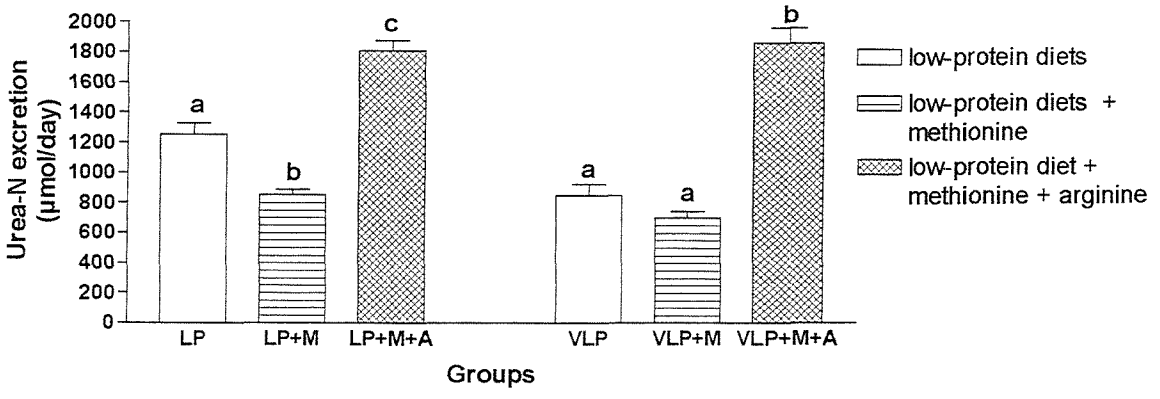


- **Results are means ± SE, n=12 per groups.**

-**Animals fed the same level of protein, and having different letter are differ significantly.**

Urea-N excretion significantly increased after dietary addition of methionine and arginine to the low-protein dietary groups (the LP+M+A and VLP+M+A groups) compared with animals fed the same levels of protein with or without methionine supplementation (see Figure 7-6).

Figure 7-6 Urea-N excretion in rats fed either low-protein diets, low-protein diets supplemented with methionine or low-protein diets supplemented with methionine and arginine-average of two days before starting treatments

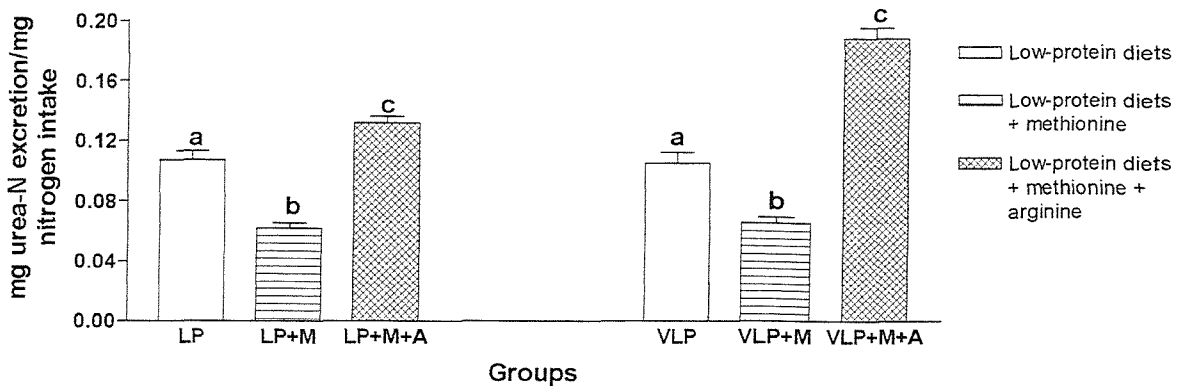


- **Results are means ± SE, n=12 per groups.**

-**Animals fed the same level of protein, and having different letter are differ significantly.**

However, addition of methionine alone to the low-protein diets resulted in a decrease in the amounts of urea-N excretion when compared with values from animals fed the same level of protein, but without amino acids supplementation. This decrease was statistically significant in animals fed the 80 g protein/kg diet. The increase in urea-N excretion, after addition of methionine and arginine to the low-protein diets, was consistent with the increase in the ratio of urea-N to nitrogen intake (Figure 7-7). The situation worsened as the dietary protein content fell from 80 g/kg to 60 g/kg of diet. This phenomenon became greater as the supply of essential amino acids (other than methionine) worsened. This may indicate that the addition of arginine increases the flow of amino acids towards the urea-cycle. Arginine is an allosteric activator of the enzyme N-acetylglutamate synthetase. Acetylglutamate is synthesised from glutamate and acetyl CoA (Kawamoto *et al.* 1982). The enzyme, N-acetylglutamate synthetase, activates carbamoyl phosphate synthase I, which is needed for synthesising carbamoyl phosphate, in the first step of ammonia detoxification. Thus, arginine could accelerate urea production by activating N-acetylglutamate synthetase. However, addition of methionine alone, with no arginine supplementation, to the low-protein dietary groups (the LP+M and VLP+M groups), reduced urea-N excretion and reduced the ratio of urea-N to N intake compared with the corresponding low-protein dietary groups with or without arginine and methionine supplementation.

Figure 7-7 The ratio of urea-N to nitrogen intake in rats fed either low-protein diets, low-protein diets supplemented with methionine or low-protein diets supplemented with methionine and arginine-average of two days before starting treatments

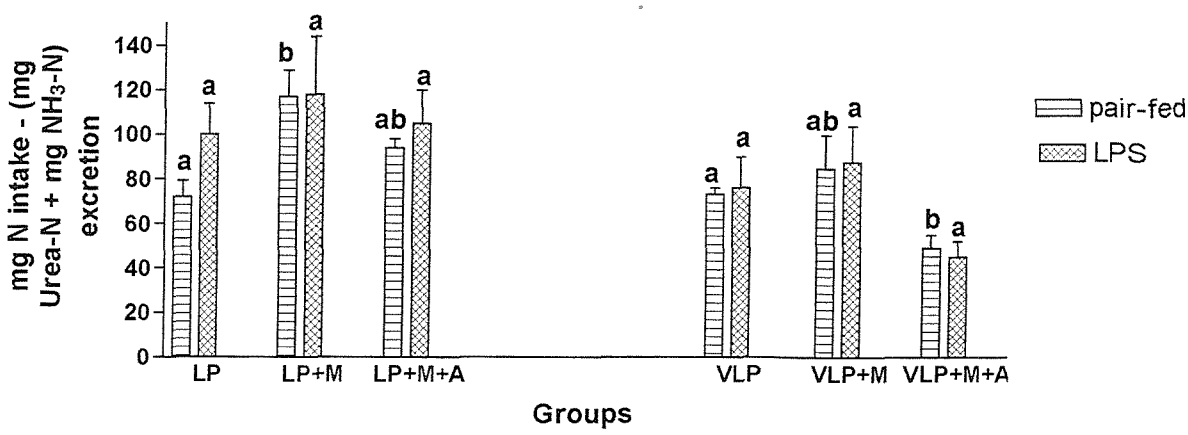


- Results are means ± SE, n=12 per groups.

-Animals fed the same level of protein, and having different letter are differ significantly.

After starting treatments, addition of methionine to the low-protein dietary group appeared to increase nitrogen retention in the LPS and pair-fed animals (Figure 7-8). The difference was statistically significant when comparing pair-fed animals fed the LP+M diet with the corresponding pair-fed animals fed the LP diet. However, in the LPS and pair-fed groups, nitrogen retention in animals fed the 60 g/kg diet was lower in arginine and methionine supplemented groups compared with animals fed the same amount of protein supplemented with methionine or without amino acids supplementation. The difference was statistically significant when comparing pair-fed animals fed the VLP+M+A diet with the corresponding pair-fed animals fed the VLP diet.

Figure 7-8 Apparent nitrogen retention in LPS-treated and pair-fed animals fed either low-protein diets, low-protein diets supplemented with methionine or low-protein diets supplemented with methionine and arginine



- Results are means ± SE, n=4 per treatment group.

- Animals having the same treatment and same protein level with different letter are differ significantly.

In addition to arginine counteracting the beneficial effect of supplementing methionine on N retention, it failed to improve organ size. Addition of arginine to the low-protein diets supplemented with methionine did not enhance the relative weight of lung, spleen, and thymus. No difference in the relative weight of lung, spleen, and thymus was found between animals the low-protein diets supplemented with arginine and methionine (Table 7.5), and animals fed the low-protein diets supplemented with methionine (Table 5.5, Chapter 5), or with methionine and tryptophan (Table 6.5, Chapter 6).

Furthermore, the relative liver weight was affected by dietary arginine supplementation in animals fed the low-protein diets supplemented with methionine. In general, there was a trend for a reduction in the liver relative weight of animals fed the LP+M+A and VLP+M+A diets compared with animals fed the same level of protein supplemented with methionine alone or with methionine and tryptophan (Table 7.17). This was consistent with the trend for a reduction in the relative liver protein found in animals fed the arginine-supplemented diets (Table 7.18).

Table 7.17 Comparison of the relative liver weight of rats fed low-protein diets, or low-protein diets supplemented with methionine, methionine and tryptophan or methionine and arginine (g/kg body weight)

	ad libitum	Pair-fed	LPS
LP	45.7 ± 1.6^{a}	42.1 ± 1.2^{ab}	52.0 ± 1.0^{a}
LP+M	55.0 ± 4.1^{bc}	45.6 ± 2.7^{ab}	57.7 ± 2.6^{ab}
LP+M+T	63.1 ± 1.5^{b}	46.7 ± 1.6^{a}	58.2 ± 0.9^{b}
LP+M+A	55.6 ± 2.7^{c}	40.8 ± 0.5^{b}	55.2 ± 1.6^{ab}
VLP	45.4 ± 1.0^{a}	38.7 ± 0.8^{a}	46.2 ± 1.3^{a}
VLP+M	64.3 ± 2.8^{bc}	47.1 ± 2.5^{bc}	60.5 ± 1.4^{b}
VLP+M+T	67.9 ± 1.8^{b}	50.9 ± 2.9^{b}	59.1 ± 2.0^{bc}
VLP+M+A	57.5 ± 2.3^{c}	40.7 ± 3.4^{c}	54.6 ± 0.9^{c}

- Results are means ± SE, n=6 per treatment group.
-Means within each column, having the same level of protein and having different letter superscripts following the number are differ significantly.

Table 7.18 Comparison of the relative liver protein of rats fed low-protein diets, or low-protein diets supplemented with methionine, methionine and tryptophan or methionine and arginine (g/kg body weight)

	ad libitum	Pair-fed	LPS
LP	6.70 ± 0.13^{a}	6.76 ± 0.49^{ab}	7.10 ± 0.24^{a}
LP+M	7.99 ± 0.30^{bc}	6.77 ± 0.25^{ab}	8.59 ± 0.37^{b}
LP+M+T	8.62 ± 0.24^{b}	7.15 ± 0.24^{a}	8.46 ± 0.27^{b}
LP+M+A	7.53 ± 0.37^{ca}	6.42 ± 0.11^{b}	7.93 ± 0.22^{b}
VLP	5.84 ± 0.17^{a}	6.20 ± 0.14^{a}	6.10 ± 0.20^{a}
VLP+M	8.09 ± 0.26^{bc}	7.31 ± 0.11^{b}	8.46 ± 0.22^{b}
VLP+M+T	8.39 ± 0.21^{b}	7.10 ± 0.17^{b}	7.92 ± 0.17^{bc}
VLP+M+A	7.51 ± 0.16^{c}	6.26 ± 0.44^{ab}	7.52 ± 0.18^{c}

- Results are means ± SE, n=6 per treatment group.
-Means within each column, having the same level of protein and having different letter superscripts following the number are differ significantly.

Finally, GSH concentrations (Table 7.6), the serum concentrations of albumin and α-1 acid glycoprotein, and the enzymes, glutathione peroxidase and reductase activities in liver, lung, spleen, and thymus, were not affected by addition of arginine

to the low-protein diets supplemented with methionine (these data can be seen in **Appendix A**).

The main conclusions that could be obtained, from the results of the present study and other studies, are the following:

1) Arginine could benefit the body after trauma or injury if it supplements a diet in which its availability limits the urea-cycle, as in animals fed a high-protein diet with a relatively low arginine content.

2) Attention should be paid to the protein quality of the diet before adding arginine. If the quality of the protein is poor, addition of arginine may cause an imbalance in the essential amino acids content of the diet, which could further deteriorate growth and cause further metabolic stress.

3) Supplements based on physiological quantities of arginine are ineffective. Most of the benefits coming from arginine supplementation, given in other studies, may be due to pharmacological effects.

4) Finally, the demand for arginine could be different depending on the type and severity of the stress exposed by the subject. For example, in trauma (e.g. bone fracture, burn), or tissue injury caused by surgery, there was a high demand of arginine to rebuild connective tissue, collagen and hair. In the case of inflammation, caused by LPS, there is no serious damage to connective tissue, arginine requirements would be less, thus, rendering dietary supplements ineffective.

CHAPTER EIGHT

8. General Discussion

The work described in the thesis presupposes that inflammation exerts specialised demands upon amino acid and protein metabolism and that these demands are different from those related to growth. A rat model was used to examine this hypothesis. The specific demands for dietary amino acid and protein to support the inflammatory response are largely unknown. In the model, the metabolic response to inflammation is impaired by feeding diets, which have increasingly deficient in protein and amino acids, judged from the point of view of requirements for growth. The amino acids considered to be important in the inflammatory response, methionine, tryptophan and arginine are added back to the diets sequentially, to identify the relative importance of each, for a maximal response. The model achieved substantial modification of the metabolic response to inflammation. When dietary protein content was decreased, the ability to increase tissue glutathione (GSH) and liver total protein content was severely impaired, lung experienced an increase in neutrophils, and the ability to increase α-1 acid glycoprotein was slightly impaired. The pattern of nitrogen and sulphur excretion was altered. Growth was severely affected and the animals became inefficient in utilising protein and energy. The results presented in this thesis show that sulphur amino acids are the most important amino acids in the group studied regarding the ability to modulate the inflammatory response and improve the growth. In general term, addition of neither tryptophan nor arginine, to the low-protein diets supplemented with methionine, exerted any further enhancement of the inflammatory response to that already seen after addition of methionine alone, according to the parameters measured in this thesis.

Calculations of the power of a study, which depend on the sample size and amount of variation within the groups, were not conducted in the present thesis. In consequence, results which showed "no statistically significant difference" may have

produced a small non-significant response. It is possible that the study missed a small effect due to small sample size and/or large variations. However, increasing the sample size was not possible within the resources available. Furthermore, it is customary in animals studies, in our laboratory and in other laboratories, to use a number of animals ranging from 4-8 per treatment group (Bauman *et al.* 1988; Hunter & Grimble, 1994; Langley *et al.* 1994; Taylor *et al.* 1992; Bagley & Stipanuk, 1995; Bella and Stipanuk, 1995). Although it is important to calculate the power of a study to detect differences based on the number of subjects before conducting any animal or human research, it is rarely used. For example, in the Journal of Nutrition, from issue no. 1, vol. 129, 1999 to issue no. 10, vol. 130, 2000, there was no rat study published which used a power calculation. Power calculations are most appropriate when the study is designed to investigate a single major outcome for which the variability is known (e.g. clinical trials).

A series of inter-related studies were used in this thesis to examine the modulatory effect of methionine, tryptophan and arginine in young growing rats. One of the limitations of the approach using inter-related studies is that there may be difficulties in repeating the same controls each time due to natural variation. However, the NP+M group was repeated three times (in chapter 5, 6 and 7) and it showed a consistency in most of the values in animals fed *ad libitum* (see Table 8.1). The variations of some of the values of the LPS, and hence in the pair-fed controls, was partly due to the variation in the appetite response to LPS between different groups of animals fed the NP+M group.

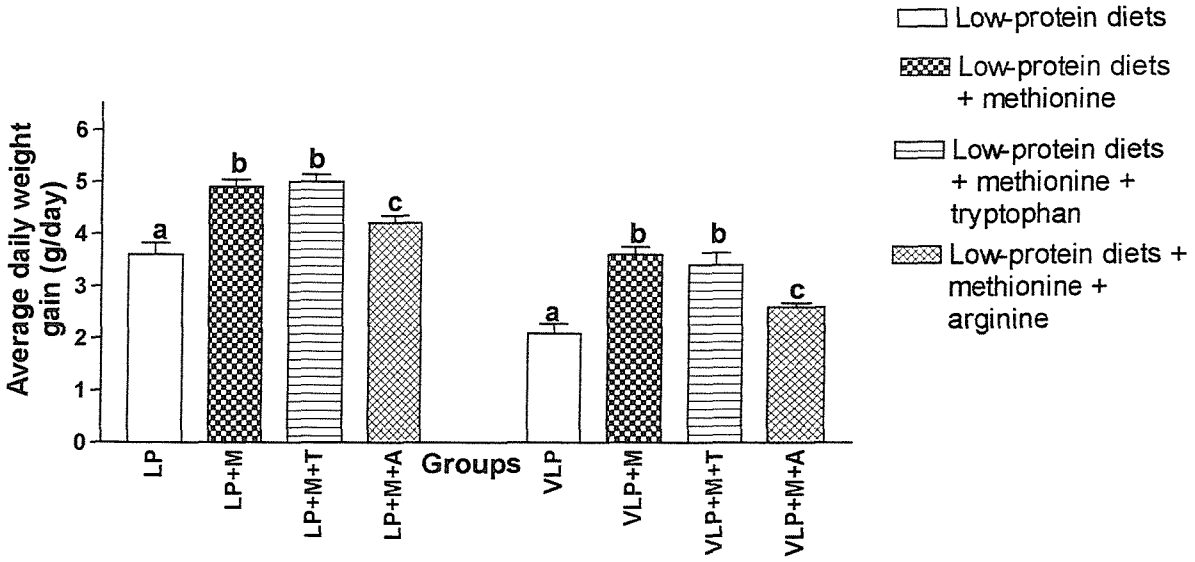
Table 8.1 Comparison between values obtained from groups of animals fed the normal-protein diet supplemented with methionine in chapters five, six and seven

	NP+M (Chapter five)	NP+M (Chapter six)	NP+M (Chapter seven)
Weight gain (g/day)	6.2 ± 0.29	6.4 ± 0.22	6.2 ± 0.19
Relative liver weight (g/kg body weight)			
ad libitum	53.0 ± 2.8	48.3 ± 1.9	46.4 ± 1.7
pair-fed	40.9 ± 2.4	44.3 ± 1.9	39.7 ± 3.6
LPS	52.0 ± 0.6	50.8 ± 1.9	51.3 ± 1.9
Liver GSH (µmol/g liver)			
ad libitum	6.18 ± 0.19	6.40 ± 0.21	6.10 ± 0.14
pair-fed	2.63 ± 0.39	3.75 ± 0.54	2.47 ± 0.35
LPS	6.84 ± 0.15	7.52 ± 0.32	6.81 ± 0.47
Liver GSH peroxidase (units/g protein)			
ad libitum	28.8 ± 1.5	29.5 ± 1.5	33.5 ± 1.5
pair-fed	28.7 ± 1.8	27.8 ± 2.2	34.2 ± 1.2
LPS	30.5 ± 1.0	31.4 ± 1.2	32.6 ± 1.3
Liver GSSG reductase (units/mg protein)			
ad libitum	20.2 ± 1.4	20.7 ± 1.2	16.2 ± 1.0
pair-fed	17.4 ± 3.4	15.3 ± 1.4	14.5 ± 2.2
LPS	16.5 ± 0.8	17.1 ± 1.6	16.0 ± 2.2
Relative liver weight (g/kg body weight)			
ad libitum	7.79 ± 0.12	7.30 ± 0.36	6.89 ± 0.24
pair-fed	6.69 ± 0.33	7.11 ± 0.17	6.20 ± 0.39
LPS	8.41 ± 0.18	8.21 ± 0.36	7.91 ± 0.25
α-1 acid glycoprotein (µg/ml)			
ad libitum	86 ± 11	86 ± 14	88 ± 10
pair-fed	90 ± 7	99 ± 8	88 ± 5
LPS	833 ± 129	854 ± 71	822 ± 91
Albumin (mg/ml)			
ad libitum	34.2 ± 0.57	35.8 ± 0.22	33.7 ± 0.21
pair-fed	33.9 ± 0.39	35.7 ± 0.87	35.0 ± 0.79
LPS	33.1 ± 0.71	34.3 ± 1.17	34.4 ± 0.33
Inorganic sulphate excretion (µmol/day)			
ad libitum	321 ± 17	316 ± 2	264 ± 29
pair-fed	254 ± 70	182 ± 36	233 ± 10
LPS	303 ± 10	236 ± 46	228 ± 17
Ammonia-N excretion (µmol/day)			
ad libitum	798 ± 20	752 ± 25	741 ± 32
pair-fed	550 ± 17	462 ± 61	511 ± 38
LPS	705 ± 77	536 ± 88	457 ± 56
Urea-N (µmol/day)			
ad libitum	10713 ± 634	10134 ± 530	10051 ± 697
pair-fed	8571 ± 226	6211 ± 449	8283 ± 425
LPS	9668 ± 1563	6970 ± 1127	8520 ± 477

8.1 Influence of dietary amino acid supplementation on utilisation of dietary protein for growth in the presence and absence of inflammation

During growth, before starting treatments, addition of methionine to the low-protein diets, with or without tryptophan or arginine supplementation, improved the average daily weight gain, food, protein, and energy efficiencies compared with the low-protein dietary groups, without amino acid supplementation (Figure 8-1 & 8-2). Addition of tryptophan to the low-protein diets supplemented with methionine did not exert further improvements in the weight gain, or in the efficiencies of food, energy and protein utilisation. Paradoxically, addition of arginine to the low-protein diets supplemented with methionine significantly reduced the weight gain, and the food, energy, and protein efficiencies of the diets compared with the low-protein diets supplemented with methionine alone or with methionine and tryptophan, despite its role as a semi-essential amino acid.

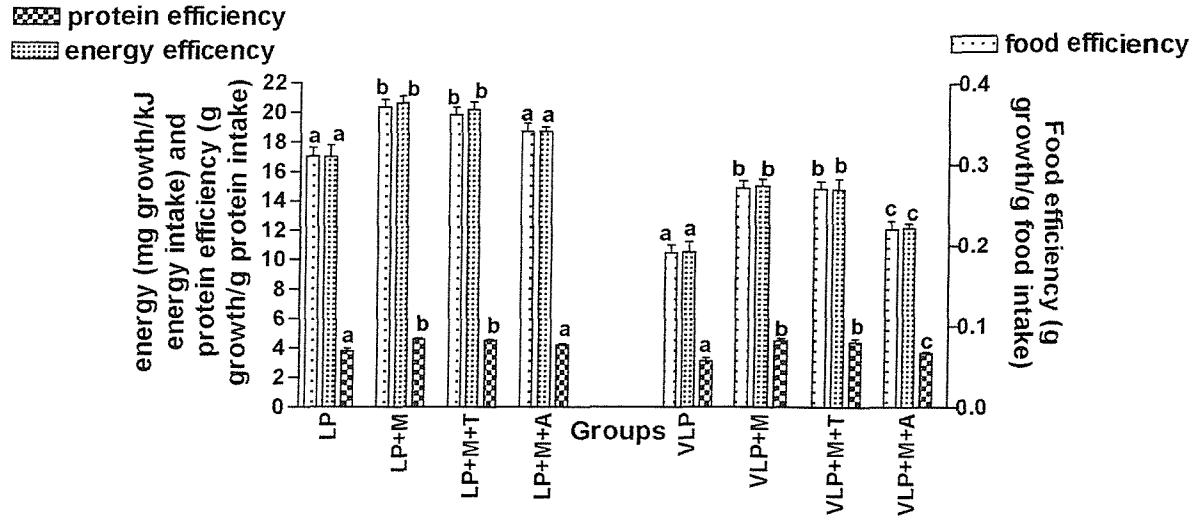
Figure 8-1 Average daily weight gain of rats fed either low-protein diets, or low-protein diets supplemented with methionine, methionine plus tryptophan or methionine plus arginine



- Results are means ± SE, n=18.

- Animals fed the same level of protein, and having different letter are differ significantly.

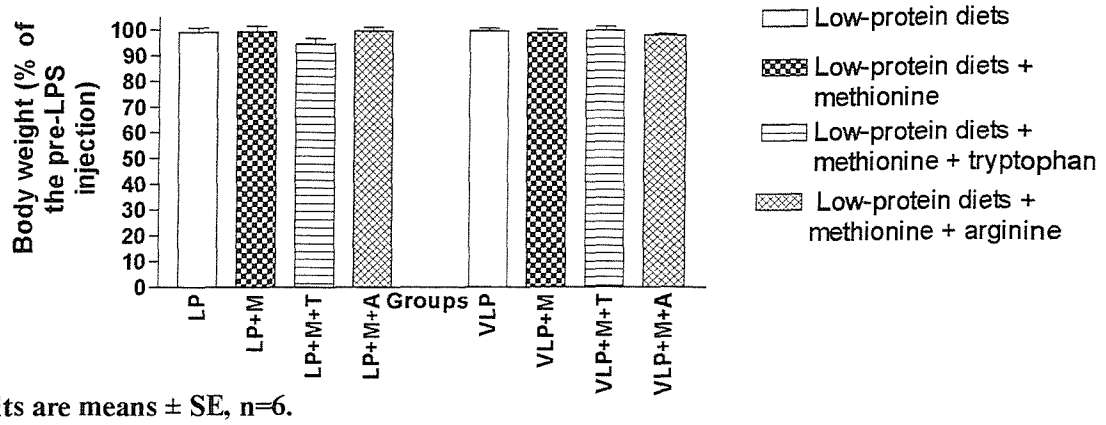
Figure 8-2 Food, energy, and protein efficiencies of rats fed either low-protein diets, or low-protein diets supplemented with methionine, methionine plus tryptophan or methionine plus arginine



- Results are means ± SE, n=18.
- Animals fed the same level of protein, and having different letter are differ significantly.

A common feature of the metabolic response to inflammatory agent is loss of appetite and weight. Infection increases the production of cytokines, which have a negative effect on the appetite centre in the hypothalamus, causing anorexia. A reduction in food intake occurred in all dietary groups undergoing an inflammatory response to LPS (see Table 4.4, chapter 4; Table, 5.3, chapter 5; Table 6.3, chapter 6; Table 7.3, chapter 7). Thus, a stunting in the weight gain of rats occurred in all dietary groups. This effect was not affected by addition of methionine, methionine and tryptophan, or methionine and arginine to the low-protein diets (Figure 8-3).

Figure 8-3 The effect of LPS treatment on body weight of rats fed either low-protein diets, or low-protein diets supplemented with methionine, methionine plus tryptophan or methionine plus arginine•



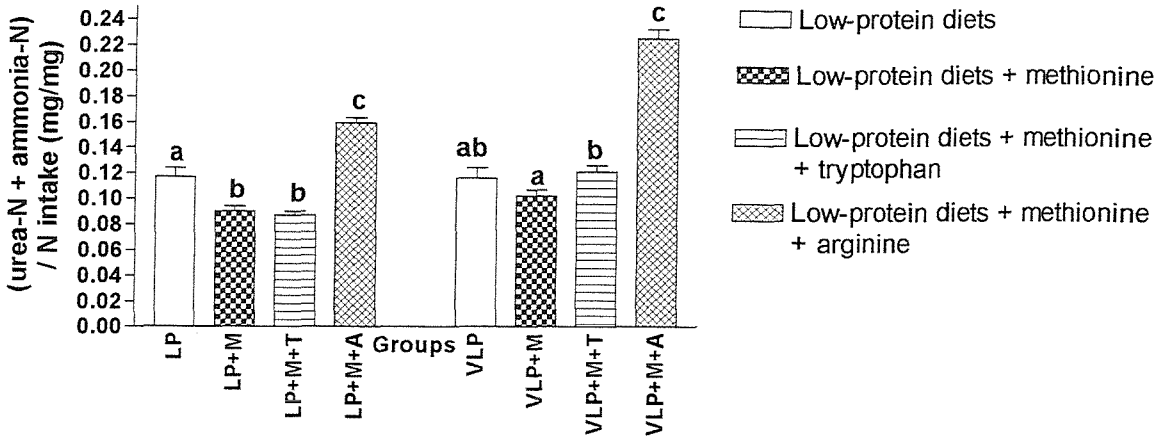
- Results are means ± SE, n=6.

• Weights are expressed as a percentage of the pre-LPS injection weight.

8.2 Influence of dietary amino acid supplementation on the partitioning of amino acids during growth

During growth, in the absence of inflammation, differences in N retention occurred. Looking at the results of the ratio of nitrogen excretion (urea-N + ammonia-N) to nitrogen intake (Figure 8-4), it can be seen that addition of arginine to the low-diets supplemented with methionine significantly increased the ratio compared with all dietary groups fed the same concentration of protein.

Figure 8-4 The ratio of nitrogen excretion (urea-N + ammonia-N) to nitrogen intake of rats fed either low-protein diets, or low-protein diets supplemented with methionine, methionine plus tryptophan or methionine plus arginine- average of two days before starting treatments



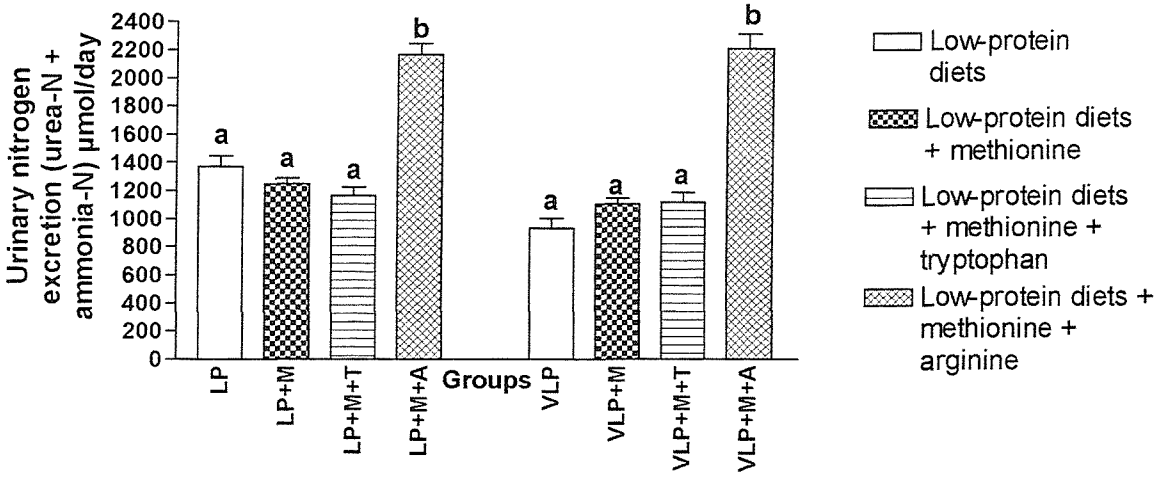
- **Results are means ± SE, n=12 per group.**
- **Animals fed the same level of protein with different letter are differ significantly.**

Addition of methionine alone or in combination with tryptophan to the low-protein diet significantly reduced the ratio (the LP+M and LP+M+T groups vs. the LP group). In animals fed the very low-protein diet, addition of methionine decreased the ratio compared with the VLP and VLP+M+T diets, indicating improved N retention in both cases. The difference was statistically significant when comparing the VLP+M group with the VLP+M+T group. However, tryptophan was unable to increase the efficiency of dietary protein utilisation further, and when diet protein content fell to 60 g/kg reversed the beneficial effects of methionine. Likewise, addition of physiological amounts of arginine to the low-protein diets supplemented with methionine opposed the beneficial effect of this latter amino acid on nitrogen retention at protein concentration of 80 and 60 g/kg diet.

Although urea-N excretion decreased, after addition of methionine, or methionine and tryptophan to the low-protein diets, compared with values from the low-protein dietary groups that received no amino acid supplementation, nitrogen excreted as ammonia increased (see chapter 4, Table 4.15; chapter 5, Table 5.14 &

chapter 6, Table 6.14). Figure 8-5 shows that nitrogen excreted as ammonia-N and urea-N was similar between the low-protein dietary groups and the low-protein dietary groups supplemented with methionine or with methionine and tryptophan. Addition of arginine to the low-protein diets supplemented with methionine significantly increased nitrogen (urea-N + ammonia-N) excreted in urine compared with other dietary groups.

Figure 8-5 Urinary nitrogen excretion (urea-N + ammonia-N) of rats fed either low-protein diets, or low-protein diets supplemented with methionine, methionine plus tryptophan or methionine plus arginine-average of two days before starting treatments



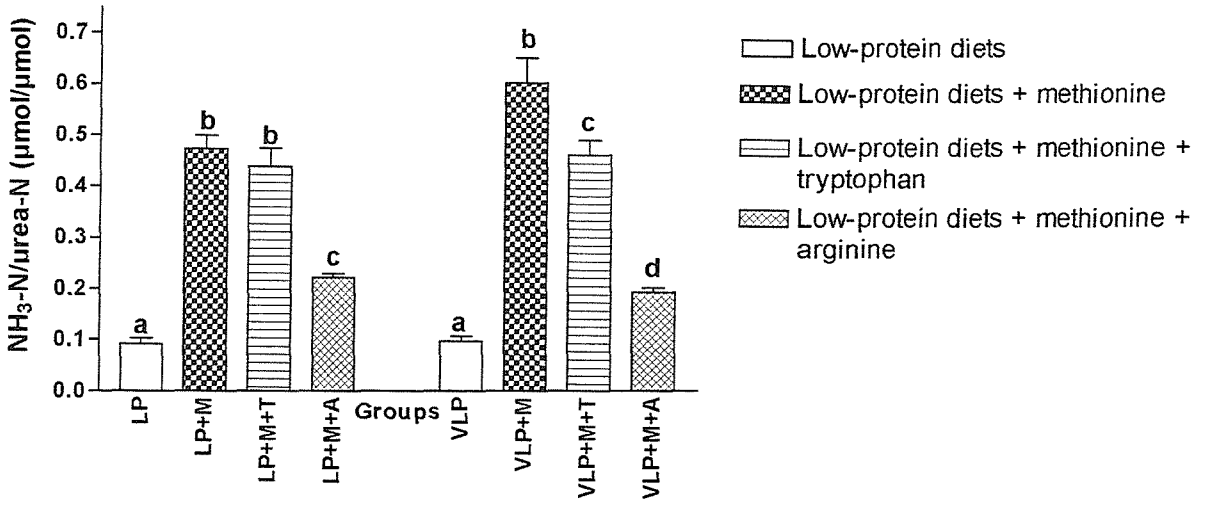
- Results are means ± SE, n=12 per group.

- Animals fed the same level of protein with different letter are differ significantly.

Despite the fact that nitrogen excretion (urea-N + ammonia-N) was similar between the low-protein dietary groups and the low-protein dietary groups supplemented with methionine or with methionine and tryptophan, addition of methionine or methionine and tryptophan to the low-protein dietary groups was accompanied by a marked increase in the proportion of urinary nitrogen excreted as ammonium (Figure 8-6). This was consistent with the study conducted by Stipanuk and her colleagues. Bella & Stipanuk (1995) have shown that total nitrogen excretion was similar between animals fed a low-protein diet (100 g/kg diet) and

animals fed the same diet, but supplemented with 24.2 g methionine/kg diet. However, as in the present study, the proportion of urinary nitrogen excreted as ammonium increased dramatically after addition of methionine. In the present studies, addition of tryptophan to the very low-protein diet supplemented with methionine, the VLP+M+T diet, caused a slight but a significant decrease in the ratio of ammonia-N to urea-N compared with the VLP+M group. This is because urea-N excretion in the VLP+M+T group increased and NH_3-N fell (not statistically significant) compared with values in the VLP+M group (see Tables 5.13 and 6.13). Addition of arginine to the low-protein dietary groups supplemented with methionine (the LP+M+A and VLP+M+A groups) significantly reduced the ratio compared with the dietary groups fed the same level of protein, but supplemented with methionine or with methionine and tryptophan (the LP+M, LP+M+T, VLP+M, and VLP+M+T groups). Not surprisingly this was due to the stimulatory effect of arginine on urea production.

Figure 8-6 The ratio of ammonia-N to urea-N excreted in urine of rats fed either low-protein diets, or low-protein diets supplemented with methionine, methionine plus tryptophan or methionine plus arginine-average of two days before starting treatments



- **Results are means ± SE, n=12 per group.**
- **Animals fed the same level of protein with different letter are differ significantly.**

So it could be concluded that methionine, as an ammoniagenic amino acid, shifts amino acids from urea synthesis to glutamine synthesis, but causes no changes in nitrogen excretion (urea-N + ammoina-N), while arginine is a ureagenic amino acid, accelerates urea excretion and increases nitrogen excretion (urea-N + ammonia-N). Thus, the metabolic characteristics of methionine, tryptophan and arginine prevent them from improving dietary protein utilisation by directly reducing urinary N excretion.

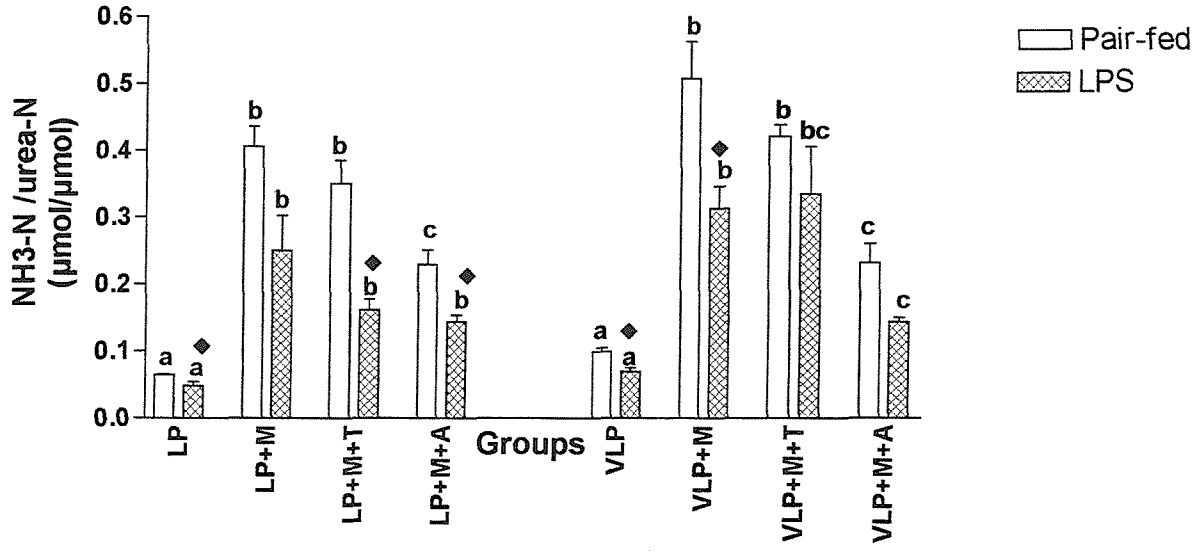
Biochemical factors also oppose the ability of methionine to improve amino acids retention. As the production of one mole of SO_4 is accompanied by two moles of H^+ (Bella & Stipanuk, 1995), the significant increase in SO_4 excretion, which was found after addition of methionine to the low-protein diets, suggests an increase in the production of H^+. As was mentioned before, during acidosis, hepatic synthesis of urea decreases, to conserve HCO_3 in order to neutralise the excess H^+ ions, and hepatic glutamine synthesis is increased (Haussinger, 1990). However, experiments conducted by Boon *et al.* (1994) have shown that in acidosis the liver does not simply shift from periportal urea synthesis to perivenous glutamine synthesis in order to achieve pH homeostasis, but that total hepatic amino acid metabolism is decreased in acidosis. Boon *et al.* (1994) and Meijer & Boon (1998) have indicated, in an vivo studies, that the transport of amino acids across the plasma membrane of the hepatocyte decreases in acidosis, and hence total hepatic amino acid oxidation decreases. Therefore, urea production decreases, and more amino acids became available for glutamine synthesis elsewhere in the body, e.g. lung, brain, skeletal muscle and adipose tissue (Watford, 1998). Consequently, in acidosis, more glutamine would be available for renal ammonia excretion and bicarbonate production. Both Boon's and Haussinger's studies agree that glutamine synthesis and ammonia excretion increase in acidosis, which supports the present results of the decrease in urea-N, increase in ammonia-N excretion, and the increase in the ratio of ammonia-N to urea-N excreted in urine, after addition of methionine to the low-protein dietary groups.

8.3 Influence of dietary amino acid supplementation on the partitioning of amino acids during inflammation

Several studies show changes in the ratio of hepatic glutamine to urea and in plasma glutamine-cystine ratio in infection, cancer and other progressive catabolic conditions (Eck *et al.* 1991; Hack *et al.* 1996; Hack *et al.* 1997). In the present studies, changes in the urinary ratio of ammonia-N to urea-N nitrogen were also found during inflammation.

The results presented in this thesis show that urea-N excretion decreased, and ammonia-N excretion and the ratio of ammonia-N to urea-N excretion were significantly increased in animals fed the low-protein diets after methionine supplementation during inflammation and after food restriction (Figure 8-7).

Figure 8-7 The ratio of ammonia-N to urea-N in LPS-treated animals and pair-fed controls fed either low-protein diets, or low-protein diets supplemented with methionine, methionine plus tryptophan or methionine plus arginine



- Results are means ± SE, n=4 per treatment group.

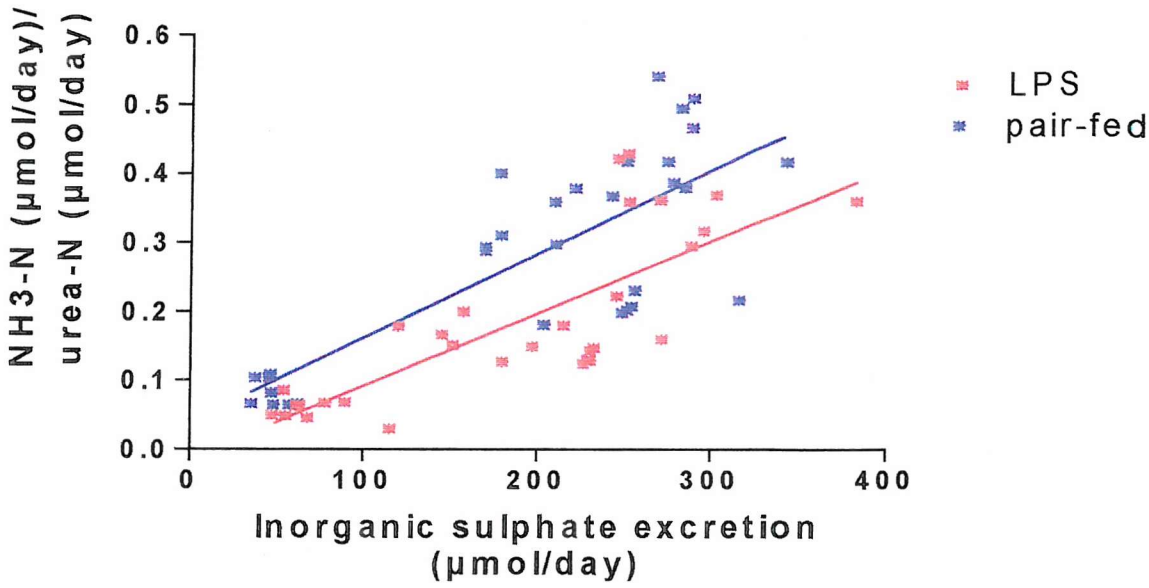
- Animals having the same treatment and same protein level with different letter are differ significantly.

- ◆ Indicates a significant difference between LPS-treated animals and corresponding pair-fed animals.

Addition of arginine to the low-protein dietary groups supplemented with methionine (the LP+M+A and VLP+M+A groups) reduced the ratio compared with the dietary groups fed the same level of protein, but supplemented with methionine or with methionine and tryptophan (the LP+M, LP+M+T, VLP+M, and VLP+M+T groups) when food intake was restricted, irrespective of whether inflammation was occurring or not. This indicates, as mentioned earlier, that the increase in nitrogen excretion after addition of arginine was mainly due to the acceleration of ureagenesis, but not ammoniagenesis.

What is more interesting is that the ratio of ammonia-N to urea-N excretion in the pair-fed controls was higher than in the corresponding LPS-treated animals. The difference was statistically significant in the LP, LP+M+T, LP+M+A, VLP, and VLP+M groups. An enhanced ratio might be expected in the LPS-treated animals in all dietary groups compared with the corresponding pair-fed controls, since inflammation might result in acidosis. In the present study, in individual rats, a strong positive correlation was found between SO_4 excretion and the ratio of NH_3-N to urea-N excreted in urine, both in LPS and in pair-fed controls (Figure 8-8). However, in the LPS-treated animals the regression line was shifted downwards suggesting that with a same level of SO_4 excretion, LPS-treated animals converts less amino acid to ammonia (lower glutamine synthesis) and more to urea than pair-fed controls. A positive correlation was also found between the ammonia-N to urea-N excretion ratio and sulphur amino acid intake, as might be expected from the characteristics of the catabolic pathway for these amino acids, but again the line of regression function shifted downwards when the inflammatory stress of LPS was applied (Figure 8-9).

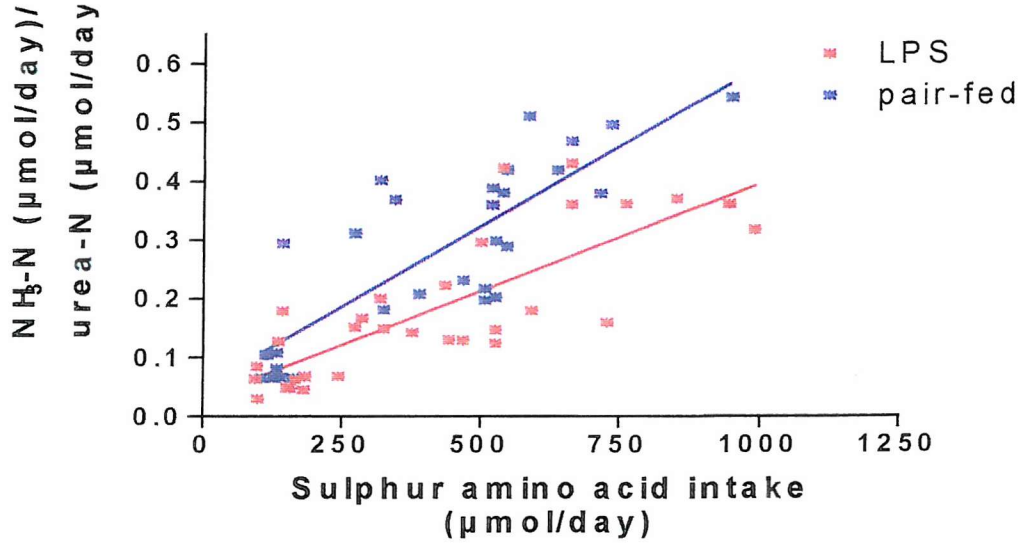
Figure 8-8 Correlation between ammonia-N to urea-N ratio and inorganic sulphate excretion in LPS-treated animals and pair-fed controls for all animals fed either low-protein diets, or low-protein diets supplemented with methionine, methionine plus tryptophan or methionine plus arginine



-LPS-treated animals n=31, p<0.000, r=0.79, r2=0.62.

-pair-fed animals, n=31, p<0.000, r=0.80, r2=0.63.

Figure 8-9 Correlation between ammonia-N to urea-N ratio and sulphur amino acid intake in LPS treated and pair-fed controls for all animals fed either low-protein diets, or low-protein diets supplemented with methionine, methionine plus tryptophan or methionine plus arginine

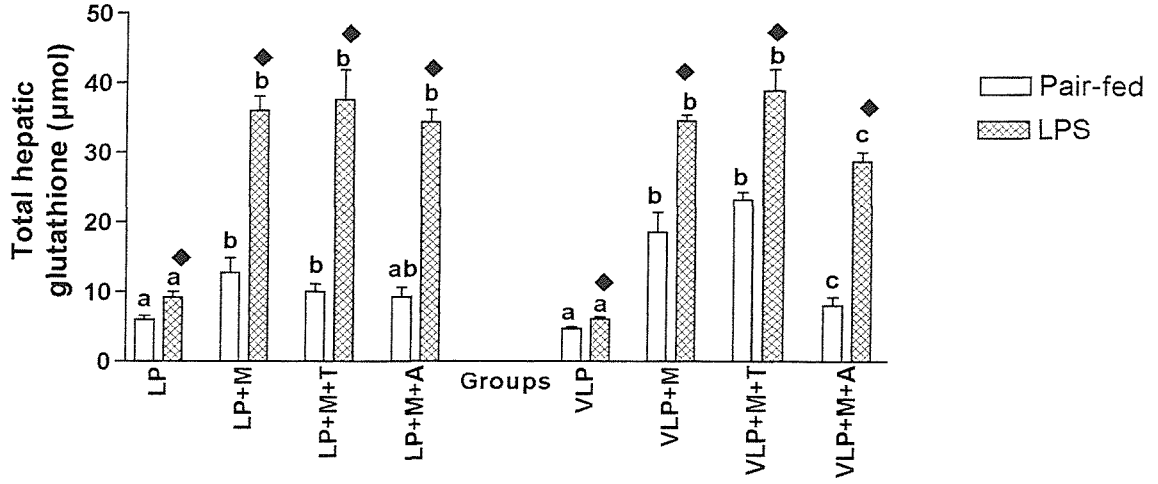


-LPS-treated animals n=31, p<0.000, r=0.79, r2=0.63.

-pair-fed animals, n=31, p<0.000, r=0.83, r2=0.69.

The decrease in the ratio of ammonia-N to urea-N excretion found in LPS-treated animals compared with the corresponding pair-fed controls, when it was correlated with a given sulphur amino acid intake (Figure 8-9), cannot be explained solely by the increase in urea-N excretion. Hepatic GSH content strongly intervenes in this relationship. To explain this, let us look back again at Figure 8-9, it can be seen that the line of regression shifts downwards when an inflammatory response was applied, and the line is widely divergent with a high sulphur amino acid intake compared with the corresponding pair-fed controls, but converges as sulphur amino acid intake decreases. This could be interpreted as an even greater suppression of ammoniagenesis at high sulphur amino acid intake during inflammation than would be expected from a given level of sulphur amino acid intake. If one relates this observation with the results of total hepatic GSH content (Figure 8-10), it can be seen that total GSH content was higher in the LPS-treated rats than the corresponding pair-fed controls, and that in the LPS-treated animals, total hepatic GSH increased to a greater extent than in the corresponding pair-fed controls, after addition of methionine, methionine and tryptophan, or methionine and arginine to the low-protein diets. In the LPS-treated animals, the dramatic increase in hepatic GSH content, after addition of methionine, means more glutamate and cysteine would be used to synthesised GSH. Thus, this could explain the decrease in the ratio of ammonia-N to urea-N excretion found in the LPS-treated animals compared with the corresponding pair-fed controls, and the wider divergence of the lines of regression between them at a high sulphur amino acid intake.

Figure 8-10 Total hepatic glutathione content in LPS-treated animals and pair-fed controls fed either low-protein diets, or low-protein diets supplemented with methionine, methionine plus tryptophan or methionine plus arginine



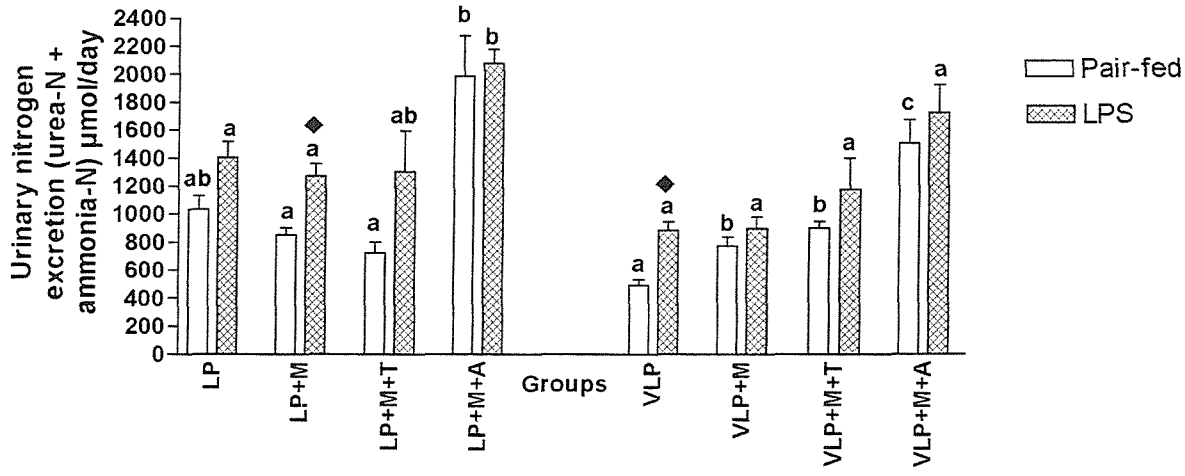
- **Results are means ± SE, n=6 per treatment group.**
- **Animals having the same treatment and same protein level with different letter are differ significantly.**
- **◆ Indicates a significant difference between LPS-treated animals and corresponding pair-fed animals.**

So it could be concluded from the results of the ratio of ammonia-N to urea-N excretion that addition of methionine to the low-protein diets allows the animals to conserve more nitrogen as glutamine instead of being converted to urea and glucose.

Hack *et al.* (1997) have shown that in HIV-infected persons, lung cancer patients, and in older people, plasma glutamine-cystine ratios (measured to give an indication of the ability of the liver to catabolised cysteine and to convert amino acids into glutamine) were lower than in healthy-young subjects. The authors conclude that cases with progressive catabolism (e.g. infection, AIDS) could be interpreted as a consequence of the low ability of liver of these patients and older people to retain the nitrogen in the amino acid pool. However, in Hack's studies, no attempt was made to measure urea and ammonia excretion in urine in catabolic conditions. In the present studies, the results of the total urinary urea-N and ammonia-N excretion shows that in LPS-treated animals no difference was found in

the total of this combined nitrogen excretion (urea-N + ammonia-N) between the low-protein dietary groups and the low-protein groups supplemented with methionine or with methionine and tryptophan (Figure 8-11), indicating that addition of methionine changes the partitioning toward glutamine synthesis, instead of urea-N, but without changing the nitrogen excretion (urea-N + ammonia-N). However, applying the inflammatory response of LPS, increased nitrogen excreted in urine (urea-N + ammonia-N) compared with the corresponding pair-fed controls. The difference was statistically significant in the LP+M and VLP groups.

Figure 8-11 Urinary nitrogen excretion (urea-N + ammonia-N) in LPS-treated animals and pair-fed controls fed either low-protein diets, or low-protein diets supplemented with methionine, methionine plus tryptophan or methionine plus arginine



- **Results are means ± SE, n=4 per treatment group.**
- **Animals having the same treatment and same protein level with different letter are differ significantly.**
- **◆ Indicated a significant difference between LPS-treated animals and corresponding pair-fed animals.**

When rats were treated with a 1µg dose of IL-6 and killed four hours later, hepatic GSH, and γ-glutamyl-cysteine synthetase activity increased, and this was accompanied by a reduction in hepatic sulphate production (Hack *et al.* 1996). In addition to this, the reduction in hepatic sulphate levels was associated with an increase in hepatic urea-production, and a decrease in hepatic glutamine to urea

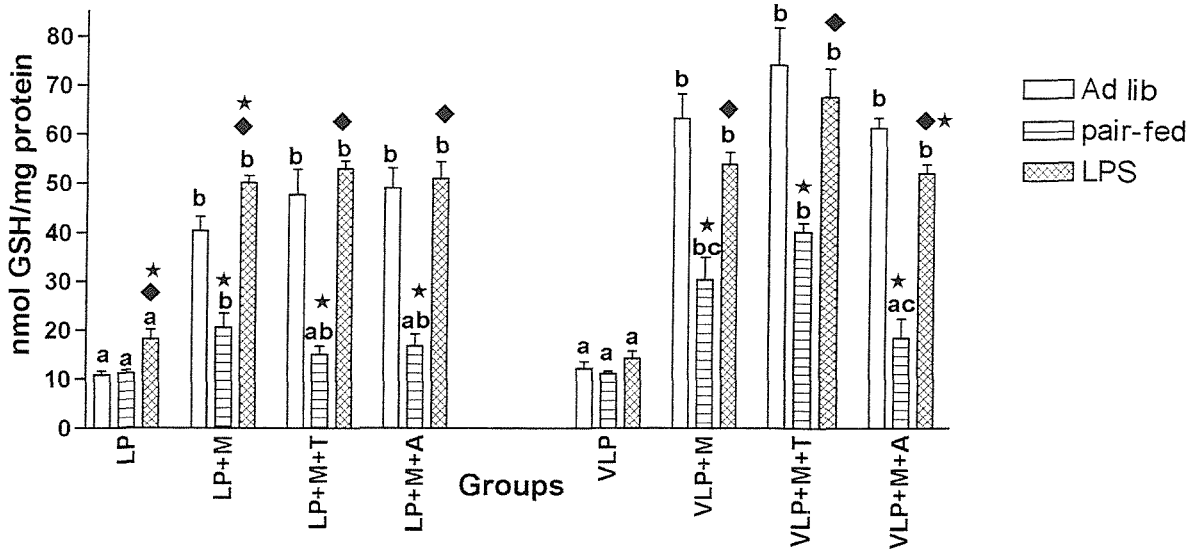
ratio. It was suggested by the authors that decreased production of H^+, due to the reduction in hepatic sulphate levels, may have increased hepatic urea production at the expense of glutamine synthesis. Increases in hepatic urea production and in the urea to glutamine ratio, associated with decreased in hepatic sulphate production, were also found in SIV-infected rhesus macaques (Eck *et al.* 1991). The same phenomena were also seen in tumor-bearing mice (Hack *et al.* 1996). However, when tumor-bearing mice were treated with daily doses of 125 or 500 μg cysteine hydrochloride monohydrate for 16 days after tumor inoculation and killed one day later, the results showed that both, mice treated with the lower dose and the higher dose of cysteine, had a higher hepatic sulphate production compared with the tumor-bearing mice unsupplemented group. Interestingly, the increase in hepatic sulphate production of mice treated with the lower dose of cysteine was accompanied by higher hepatic glutamine to urea ratios and lower hepatic urea levels compared with the tumor-bearing mice unsupplemented group. In contrast to the liver, GSH levels decreased and sulphate levels increased in skeletal muscle of tumor-bearing mice unsupplemented group. Cysteine treated tumor-bearing rats have shown an increase in muscle GSH levels (not statistically significant), but had no effect on sulphate levels compared with tumor-bearing rats unsupplemented group. The increase in the hepatic glutamine to urea ratio, and the decrease in hepatic urea production found in tumor-bearing mice treated with the lower dose of cysteine was consistent with the present study, in which the urea-N excretion decreased and urinary ratio of ammonia-N to urea-N excretion increased, after addition of methionine to the low-protein diets, during inflammation.

8.4 The partitioning of amino acids between glutathione and protein in liver and inorganic sulphate excretion

It is apparent in the studies described in this thesis that protein deficiency exerts major effect on the ability of rat to synthesise major sulphur containing products of amino acid metabolism, such as protein, GSH and SO_4. The measurements made in the programme of research give some insight into

biosynthesis of these products by the liver. The results suggest that protein deficiency does not affect the ability to synthesise/maintain all of these products equally, when animals are exposed to normal or restricted feeding or are undergoing an inflammatory response. Equally, when the low-protein diets are supplemented with methionine alone or in combination with tryptophan, or arginine, the partitioning of amino acid sulphur into these major products is not equal. Examining of the ratio of hepatic GSH to protein and hepatic GSH to urinary SO_4 give some insight into the changes in partitioning which occur. The results of the ratio of GSH to protein in liver (Figure 8-12) indicates that the increase in hepatic GSH and protein content, after addition of methionine to the low-protein diets, was not in similar proportions. Addition of methionine to the low-protein diets alone or in combination with tryptophan or arginine increased the ratio of GSH to protein in liver in all treatment groups. However, the increase in the ratio was markedly higher in the LPS-treated rats and *ad libitum*-fed animals than in those on a restricted food intake. Addition of tryptophan to the very low-protein diet supplemented with methionine slightly increased (not statistically significant) the ratio over that seen in animals fed the very low-protein diet supplemented with methionine, or with methionine and arginine. The increase in the ratio of GSH to protein in liver after addition of methionine should be expected if we take into our consideration that GSH synthesis requires only cysteine, glycine, and glutamate, while protein synthesis requires all amino acids, essential, which are present in an inadequate amounts in the low-protein diets (the LP and VLP diets), semi-essential, and non essential amino acids.

Figure 8-12 The ratio of glutathione to protein in liver of rats fed either low-protein diets, or low-protein diets supplemented with methionine, methionine plus tryptophan or methionine plus arginine



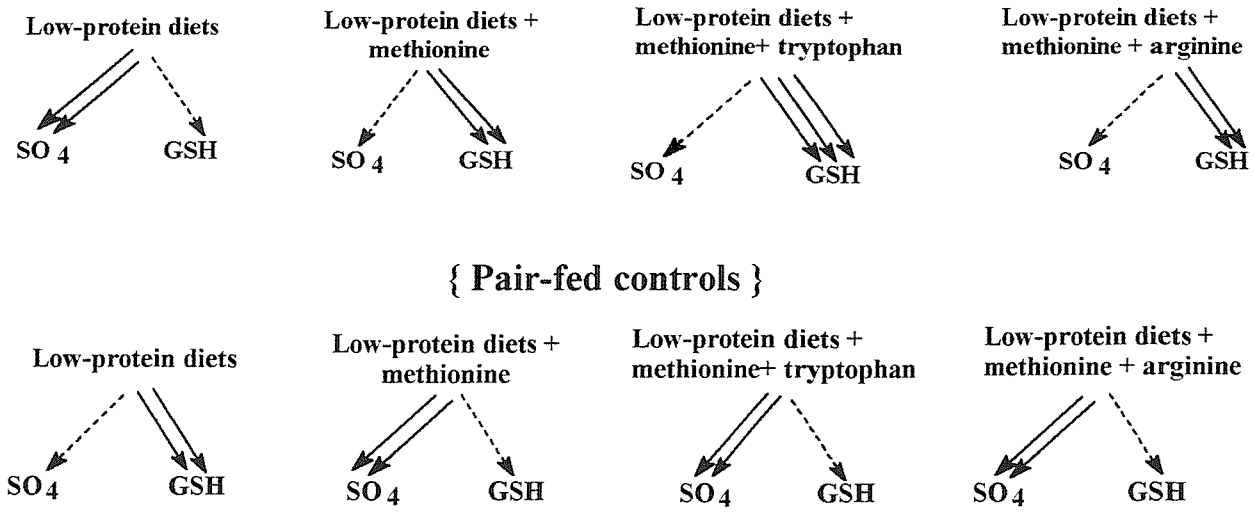
- **Results are means ± SE, n=6 per treatment group.**
- **Animals having the same treatment and same protein level with different letter are differ significantly.**
- **◆ Indicates a significant difference between LPS-treated-group and corresponding pair-fed group. ⋆ indicates a significant difference between *ad libitum* group and corresponding pair-fed or LPS-treated groups.**

Although addition of methionine to the low-protein diets increased hepatic GSH concentration and SO_4 excretion, the increase was not proportional. In animals undergoing an inflammatory response and in *ad libitum*-fed animals, addition of methionine to the low-protein diets alone or in combination with tryptophan or arginine, increased the ratio of hepatic GSH to SO_4 excretion (see Tables 4.16, 5.15, 6.15, 7.12 & Figure 8-13 for illustration). The presence of additional tryptophan to the low-protein diets supplemented with methionine increased the ratio to a greater extent than after addition of methionine or methionine and arginine, but these differences were not statistically significant. A good example of this phenomenon can be seen in animals consuming the same amount of food (thus ruling out food intake as a variable) for example between LPS-treated animals fed the VLP diet in chapter 4 (Table 4.16), fed the VLP+M diet in chapter 5 (Table 5.15), and fed the

VLP+M+T diet in chapter 6 (Table 6.15). Another example is between animals fed *ad libitum* the LP+M diet in chapter 5 (Table 5.15), fed the LP+M+T diet in chapter 6 (Table 6.15), and fed the LP+M+A diet in chapter 7 (Table 7.12).

However, when food intake was restricted, the pattern of the partitioning was completely different from that seen in the animals fed *ad libitum* and in LPS-treated animals. In the pair-fed controls, the ratio of hepatic GSH to SO_4 excretion in the low-protein dietary groups was higher than in the pair-fed controls given the same levels of protein, but with methionine, methionine and tryptophan or with methionine and arginine supplementation (Figure 8-13).

Figure 8-13 The partitioning of sulphur amino acids between hepatic glutathione and inorganic sulphate excretion



Key: flow through metabolic pathways

..... ▸ **Low.**

——— ▸ **Increased in proportion to number of arrows.**

So it could be concluded that addition of methionine to the unstressed, or to the LPS-treated animals, relatively increased the flow of cysteine towards the pathway of hepatic GSH. However, when food intake is restricted by pair feeding, addition of methionine result in a relative decrease in partitioning of cysteine towards the

pathway of GSH. In the low-protein group, addition of methionine increased hepatic GSH concentration by 258%, 208, and 70% for the *ad libitum* fed, LPS-treated, and pair-fed animals respectively, and in the very-low protein group, addition of methionine increased hepatic GSH concentration by 414%, 304% and 161% for the *ad libitum* fed, LPS-treated, and pair-fed animals respectively.

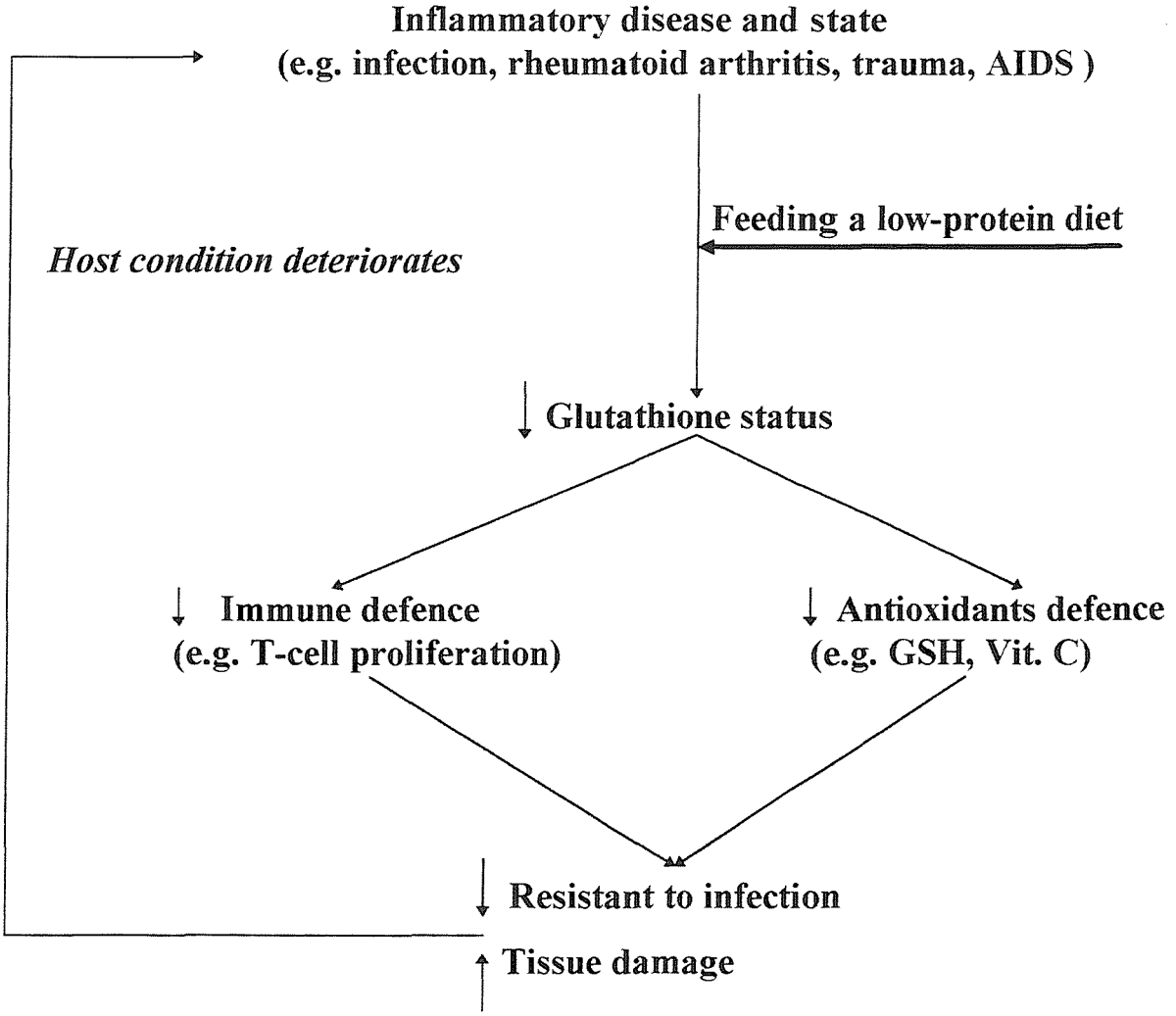
Because SO_4 excretion was similar between the LPS-treated and the corresponding pair-fed controls, after addition of methionine, methionine and tryptophan, or methionine and arginine to the low-protein groups (see Tables 5.12, 6.12 & 7.9), this may indicate that when the inflammatory response was applied, most of the additional sulphur amino acids released from muscle are mostly used to synthesised hepatic GSH and protein.

8.5 Potential benefits of sulphur amino acid supplementation in immuno-nutrition

Without doubt the largest modulatory influence was achieved with methionine supplementation in the present studies and as a consequence one is led to believe that the main limiting amino acids for the acute phase response, in the animal model employed in the thesis, are methionine and cysteine. The results presented in chapter 4, show that all treatment groups (*ad libitum*, pair-fed, and LPS-treated animals), were unable to increase hepatic GSH concentration to the same level as in animals fed the normal-protein diet. LPS-treated animals given the low-protein diets were unable to enhance GSH concentrations in lung, spleen, and thymus to the same level as in the normal-protein group. Furthermore, animals given the low-protein diets fail to enhance lung, spleen, and thymus GSH concentrations compared with the corresponding pair-fed controls. The low GSH concentrations in liver, lung, spleen, and thymus, found in the low-protein dietary groups after LPS-treatment, could be an indication of the reduction in the ability to scavenge free radicals. Moreover, the inability to enhance GSH concentrations in various organs, is an indication of a low availability of cysteine in these organs. Both cysteine and GSH have been shown to be important in optimising T-cells and macrophage immune

function (Gmunder, *et al.* 1990 & 1991; Robinson *et al.* 1993; Kinscherf *et al.* 1994). GSH is also an important compound needed to maintain the function of other antioxidant compounds. GSH is used by several transhydrogenases to maintain other antioxidant compounds in their reduced form, such as vitamins C and α-tocopherol (Martensson, *et al.* 1993; Pascoe & Reeds, 1989). Figure 8-14 shows the possible consequences of feeding a low-protein diet with respect to the relationship between some inflammatory diseases (e.g. infection) and GSH status.

Figure 8-14 Relationship between some inflammatory diseases, glutathione and host condition

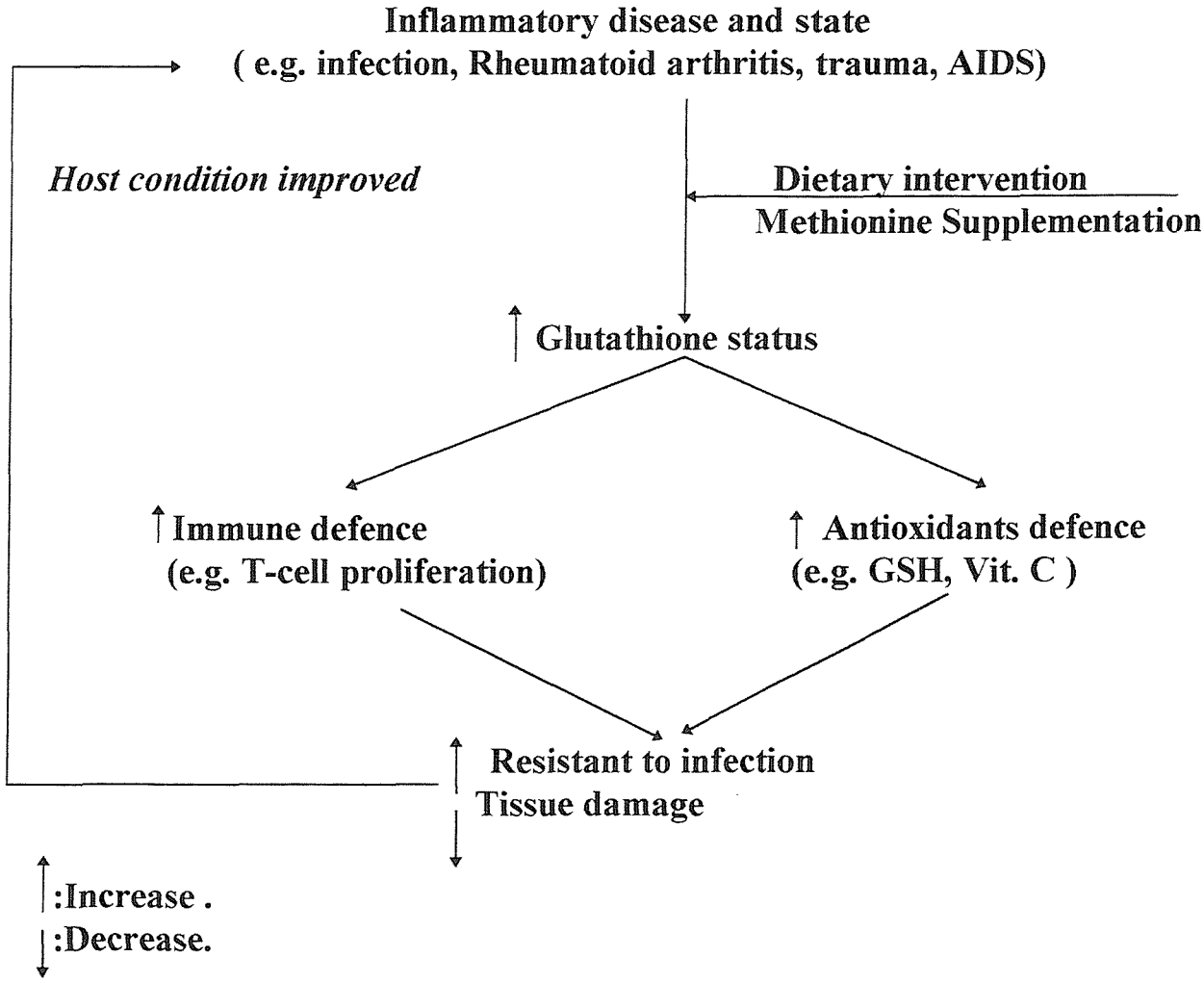


↓: Decrease.

↑: Increase.

In animals fed the low-protein diets, a significant increase in hepatic GSH concentration was achieved by adding a single amino acid, namely methionine. Addition of methionine to the low-protein dietary groups, allowed animals to enhance their lung GSH concentration, and maintain their spleen, and thymus GSH concentrations during an inflammatory response. Furthermore, in animals exposed to the inflammatory response of LPS, methionine supplementation increased hepatic protein content and increased the serum concentration of α-1 acid glycoprotein over that seen in animals fed the low-protein diets, without amino acid supplementation. The evidence from the lung neutrophil data suggest that methionine, by enhancing GSH status within the body, could increase the ability of the host to scavenge free-radicals, and improve T-cell proliferation and macrophage function (see Figure 8-15).

Figure 8-15 Relationship between some inflammatory diseases and glutathione, and host condition. The possible role of methionine in this relationship



8.6 Enhanced glutathione status in clinical trials

Impaired GSH status is implicated in many diseases such as cancer, Alzheimer's disease, atherosclerosis and AIDS. Malnutrition, secondary to cancer, AIDS, and many other inflammatory diseases are reported in many patients, which may increase host susceptibility to infection and increase free-radical production. Thus, the need for pro-GSH agents, to be used as a part of treatment in such patients, is necessary. Because cysteine is unstable in its reduced form, and has been reported to be toxic in vivo and in vitro studies (Estrela *et al.* 1983; Nishiuch, *et al.* 1976; Harber, 1970; Olney & Ho, 1970), and because GSH is mostly degraded in the extracellular compartment, due to the presence of the enzyme γ-glutamyl cysteine transferase, several compounds have been used as a cysteine-delivery agents. L-2-oxothiazalidine-4-carboxylate (OTC) and N-acetyl-L-cysteine (NAC), are two of the most common compounds used to serve as a cysteine-delivery agents (Sen, 1997). OTC is an analog of 5-oxoproline in which the 4-methylene moiety is replaced by sulphur (Meister *et al.* 1986). OTC has been shown to be an excellent substrate of the intracellular enzyme 5-oxoprolinase (Williamson & Meister, 1981; Williamson & Meister, 1982). The enzyme converts OTC to S-carboxy-L-cysteine which is rapidly hydrolysed to yield cysteine. The other cysteine-delivery agent, NAC, once entering the cell, is rapidly hydrolysed to yield cysteine.

Recently, many clinical trials were carried out using OTC and NAC to enhance GSH status. OTC supplementation to malnourished rats increase lung GSH concentration and at the same time protected the animals against pulmonary oxygen toxicity (Taylor, 1992). Limuro *et al.* (2000) have shown that necrosis, inflammation, TNF-α and NFκB, were all elevated in rats with alcoholic-liver injury, caused by feeding ethanol containing diets enterally for 4 weeks. These effects of ethanol were blocked by giving OTC. In asymptomatic HIV-infected patients, intravenous or oral OTC treatment increased GSH level in whole blood (Kalayjian, *et al.* 1994; Barditch-Crovo, 1998). OTC supplementation has also been shown to be protective against a lethal dose of acetaminophen given to mice (Williamson *et al.* 1982).

From an antioxidant point of view, NAC has been shown to act as a cysteine scavenger (Aruoma *et al.* 1989), and act as a cysteine-delivery agent for GSH synthesis (Issels *et al.* 1988). NAC and OTC treatments have been shown to reduce the number of days with acute lung injury in adult respiratory distress syndrome (Bernard *et al.* 1997). Low GSH status is usually seen in AIDS patients with wasting syndrome, especially in their T-lymphocyte. It has been shown that the survival time was improved in AIDS patients who maintained higher GSH level in their CD $^{+4}$ T-cells than in those with lower levels, and that the survival time of patients treated with NAC was higher (Herzenberg *et al.* 1997).

To conclude, the studies reported in this thesis show that the amino acid and protein demand for growth and for a maximal acute phase response (judge against that of the 180 g/kg casein diet supplemented with/without methionine) are not qualitatively but quantitatively different. However the presence or absence of the supplementary amino acids alters the partitioning of sulphur and other amino acid into various end products, in a different way during inflammation to that occurring in growth. Furthermore, from the clinical trial studies and from the present study, it can be concluded that sulphur amino acids are important immuno-nutrient compounds both for malnourished subject and for patients with chronic catabolic diseases. In general, the importance of sulphur amino acids in clinical nutrition is underestimated. Patients with inflammatory disease and also doctors, should know the benefits of feeding sulphur amino acids, which types of food are rich in sulphur amino acids (e.g. liver, egg, whey protein), and why they are important. Putting this message into practice is the responsibility of nutritionist and dietician.

Appendix A

Table A.1 Glutathione peroxidase activity in liver, lung, spleen, and thymus of rats fed graded casein content but with identical amounts of sulphur amino acids and arginine (units/g protein)

Dietary groups g protein/kg diet	NP+M 180+M	LP+M+A 80+M+A	VLP+M+A 60+M+A	two-way ANOVA p-value	 F ratio
Liver					
ad libitum	33.5 ± 1.5^{a}	35.7 ± 2.7^{ab}	43.3 ± 1.9^{b}	D=0.001	F=9.60
pair-fed	34.2 ± 1.2^{a}	32.9 ± 1.0^{a}	33.6 ± 1.8^{a}*	T=0.038	F=3.72
LPS	32.6 ± 1.3^{a}	35.5 ± 2.9^{ab}	42.8 ± 1.8^{b}♠	I=0.057	F=2.62
Lung					
ad libitum	65.9 ± 3.1^{a}	65.6 ± 2.3^{a}	62.9 ± 2.1^{a}	D=0.316	F=1.20
pair-fed	59.5 ± 3.0^{a}	62.8 ± 3.6^{a}	66.1 ± 1.9^{a}	T=0.479	F=0.76
LPS	59.5 ± 1.9^{a}	62.1 ± 1.0^{a}	65.5 ± 2.9^{a}	I=0.344	F=1.17
Spleen					
ad libitum	66.2 ± 5.0^{a}	64.7 ± 1.5^{a}	66.3 ± 2.0^{a}	D=0.229	F=1.57
pair-fed	63.3 ± 2.9^{a}	65.9 ± 0.8^{a}	66.4 ± 1.6^{a}	T=0.677	F=0.40
LPS	62.0 ± 2.6^{a}	60.6 ± 2.8^{a}	68.9 ± 0.4^{a}	I=0.548	F=0.78
Thymus					
ad libitum	69.9 ± 5.4^{a}	70.8 ± 1.1^{a}	65.3 ± 4.7^{a}	D=0.809	F=0.21
pair-fed	71.7 ± 4.9^{a}	64.2 ± 5.5^{a}	73.4 ± 2.3^{a}	T=0.909	F=0.10
LPS	68.8 ± 1.5^{a}	69.7 ± 3.4^{a}	71.5 ± 4.4^{a}	I=0.442	F=0.97

-Results are presented as mean ± standard error of the mean, n=4 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means with in each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

Table A.2 Glutathione reductase activity in liver, lung, spleen and thymus of rats fed graded casein content but with identical amounts of sulphur amino acids and arginine (units/mg protein)

Dietary groups g protein/kg diet	NP+M 180+M	LP+M+A 80+M+A	VLP+M+A 60+M+A	two-way ANOVA p-value	 F ratio
Liver					
ad libitum	16.2 ± 1.1 [a]	19.6 ± 2.5 [ab]	27.4 ± 0.8 [b]	D=0.000	F=11.07
pair-fed	14.5 ± 2.2 [a]	19.2 ± 3.8 [a]	18.8 ± 3.6 [a]	T=0.072	F=2.91
LPS	16.0 ± 2.2 [a]	22.2 ± 0.8 [a]	26.7 ± 1.1 [b]	I= 1.09	F=0.383
Lung					
ad libitum	17.9 ± 1.54 [a]	18.4 ± 1.6 [a]	18.2 ± 1.3 [a]	D=0.337	F=1.13
pair-fed	15.7 ± 1.13 [a]	19.0 ± 1.3 [a]	18.2 ± 1.2 [a]	T=0.364	F=1.05
LPS	16.1 ± 1.50 [a]	16.3 ± 1.0 [a]	17.5 ± 1.0 [a]	I=0.725	F=0.51
Spleen					
ad libitum	17.1 ± 3.2 [a]	16.6 ± 1.5 [a]	20.1 ± 1.6 [a]	D=0.079	F=2.79
pair-fed	15.4 ± 0.6 [a]	17.8 ± 0.9 [a]	18.7 ± 0.9 [a]	T=0.748	F=0.29
LPS	16.8 ± 1.0 [a]	15.7 ± 0.8 [a]	18.5 ± 1.6 [a]	I=0.781	F=0.44
Thymus					
ad libitum	15.6 ± 1.8 [a]	16.8 ± 1.4 [a]	18.7 ± 1.3 [a]	D=0.140	F=2.12
pair-fed	17.4 ± 1.3 [a]	15.1 ± 1.2 [a]	17.9 ± 0.8 [a]	T=0.221	F=1.60
LPS	18.2 ± 0.5 [a]	17.9 ± 1.5 [a]	19.7 ± 1.8 [a]	I=0.781	F=0.44

-Results are presented as mean ± standard error of the mean, n=4 per treatment group. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means with in each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

Table A.3 Serum α-1 acid glycoprotein concentration of rats fed graded casein content but with identical amounts of sulphur amino acids and arginine

Dietary groups g protein/kg diet	NP+M 180+M	LP+M+A 80+M+A	VLP+M+A 60+M+A	two-way ANOVA p-value	 F ratio
α-1 acid glycoprotein (μg/ml)					
ad libitum	88 ± 10 [a]	98 ± 4 [a]	93 ± 7 [a]	D=0.747	F=0.29
pair-fed	88 ± 5 [a]	95 ± 6 [a]	97 ± 8 [a]	T=0.000	F=250.6
LPS	822 ± 91 [a]♠*	842 ± 102 [a]♠*	896 ± 37 [a]♠*	I=0.934	F=0.21

-Results are presented as mean ± standard error of the mean, n=4 per treatment group for animals fed the NP+M diet, and n=6 per treatment group for animals fed the LP+M+A and the VLP+M+A diets. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means with in each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

Table A.4 Serum albumin concentration of rats fed graded casein content but with identical amounts of sulphur amino acids and arginine

Dietary groups g protein/kg diet	NP+M 180+M	LP+M+A 80+M+A	VLP+M+A 60+M+A	two-way ANOVA p-value	 F ratio
Albumin (mg/ml)					
ad libitum	33.7 ± 0.21 [a]	32.9 ± 0.89 [a]	29.8 ± 0.74 [b]	D=0.000	F=23.74
pair-fed	35.0 ± 0.79 [a]	33.7 ± 0.82 [ab]	31.7 ± 0.70 [b]	T=0.013	F=4.94
LPS	34.4 ± 0.33 [a]	31.5 ± 0.19 [b]♠	29.0 ± 0.69 [c]♠	I=0.548	F=0.78

-Results are presented as mean ± standard error of the mean, n=4 per treatment group for animals fed the NP+M diet, and n=6 per treatment group for animals fed the LP+M+A and the VLP+M+A diets. D< refer to p-value for diet, T< refer to p-value for treatment, I< refer to p-value due to interaction between diet and treatment. Means with in each row having different letter superscripts following the number differ significantly. * indicates a significant difference between *ad libitum* group and pair-fed or LPS-treated groups. ♠ indicates a significant difference between pair-fed and LPS-treated groups.

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