Chapter 3.

3. TMD and Changes in Biochemistry, Amino and Organic Acid Metabolism.

3.1. Introduction.

Chapter 1 details the pilot study data of 35 chronic RDC/TMD type 1a pain patients, which found an association with multi-organ symptoms and a history of urinary tract infection (McGregor et al, 1992). These patients had an increased prevalence of elevated midstream urinary staphylococcal counts \((P<0.02)\) compared with age and sex matched controls (McGregor et al, 1993) and preliminary tests showed an association between the toxicity of these organisms and pain expression. Chapter 2 confirmed the pilot study association between RDC/TMD type 1a pain and multiple body symptoms and infectious events, and also established an association between TMD and alterations in cognitive functions. However multiple regression analysis revealed that TMD symptoms were clustered with a distinct group symptoms that had an increased prevalence in patients with multiple body symptoms but were unrelated to the majority of symptoms that also increased in the polysymptomatic patients. This suggests that the cluster of symptoms associated with TMD symptoms have a distinct molecular basis, which is consistent with the hypothesis proposed by Plesh et al (1996).

Russell et al (1989) found alterations in serum amino acids and proposed a serotonin-based hypothesis for chronic pain (Russell et al, 1992). Conversely, alterations in jaw muscle tone, one feature of TMD, has been associated with alterations in dopaminergic activity (Lynch et al, 1961; Malkin, 1964; Evans, 1965; Shapiro et al, 1965; Hiatt & Schwartz, 1966; Kraak, 1967; Bell, 1969; Sunden Kuronen et al, 1983a, 1983b; Pertoutka et al, 1988). Infections and infection-associated cytokine activity are associated with changes in amino acid metabolism (Mortimore & Poso, 1987; Hasselgren, 1995; Jeevanandam, 1995) and painful muscle has been found to have reduced total protein and RNA levels (Young, 1970; Pacy 1988; Bowles et al, 1993). In CFS patients, symptom expression has been associated with increased RNase-L activity, the activation of which is usually an cytokine-associated event (Suhaldonik et al, 1994; 1997). This increased RNase-L activity is associated with reduced RNA levels and subsequently reduced protein synthesis and a reduction in muscle protein and RNA as already noted in painful muscle (Young, 1970; Pacy 1988; Bowles et al, 1993). These data provide a body of evidence that suggests that evaluation of amino acid metabolism associated with infectious symptoms in TMD patients be warranted.

This chapter assesses standard blood cell and biochemistry, amino acid metabolism and alterations in RNase-L, soluble interleukin-2 receptor (sIL-2r) and interleukin-6 (IL-6) in
relationship to TMD patients. The data was obtained from; a) a study group with defined RDC/TMD type 1(a) (Le Resche et al, 1992); b) a second study group with defined chronic fatigue syndrome (Sharpe et al, 1991) as in Chapter 2; and c) a third CFS study group, independently obtained, to assess the association between TMD and RNase-L, sIL-2r and IL-6. The aim of evaluation of the CFS patient groups and the defined RDC/TMD type 1a group is to determine the relationship between TMD symptom expression and the biochemistry and to reduce the potentially confounding factors that may relate to symptom expression in other parts of the body as demonstrated for symptom expression in chapter 2.

3.2. Literature Review.

3.2.1. Overview of Amino Acid Metabolism.

Figure 3.1 shows an overview of human amino acid metabolism. Dietary protein, microbial produced amino and biogenic amine compounds as well as protein components of human excretory products, such stomach and pancreatic enzymes, are absorbed from the gut. The amine compounds enter the portal blood circulation where they are delivered to the liver (pathway 1 figure 3.1). The liver detoxifies the biogenic amines and processes the amino acids. Some amino acids are stored in the liver whilst others are released into the circulation where they are transported into the peripheral tissues for usage in protein synthesis and energy provision (pathway 3, Figure 3.1). Utilisation of the amino acids for energy provision occurs by degradation into the glycolytic and citric acid pathways, with the excess resultant nitrogen entering the urea cycle for conversion to urea (pathway 2, Figure 3.1). Excess nitrogen (as urea) is excreted from the kidney (pathway 4, Figure 3.1). During normal metabolism, protein degradation is balanced by protein synthesis and excess nitrogen from the tissues is returned to the liver as either alanine or glutamine (Lehninger et al, 1993). However during periods of increased energy requirement, such as infection, increased exercise, stress or other illness, muscle, adipose tissue and connective tissues undergo increased proteolysis to provide amino acids for the period of increased energy demand (Mortimore & Poso, 1987).

Disposal of the excess ammonia and nitrogen to the liver from catabolism of amino acids within muscle occurs via release of alanine and glutamine from muscle (Figure 3.2). This reaction is mediated by the enzyme alanine aminotransferase (ALT) in both muscle and liver and is intimately involved with glycolysis and gluconeogenesis in both muscle and liver (Lehninger et al, 1993).
Figure 3.1. Overview of Human Amino Acid Metabolism.

1. Protein and amino acids of dietary, host secretory and microbial origin are absorbed from the gastrointestinal tract.
2. The basic biochemical mechanisms that result in urea production and ammonia removal.
3. Protein synthesis and proteolysis regulate the serum amino acid levels with the muscle, adipose and connective tissue acting as the reservoirs of amino acids.
4. Urea is transported to the kidney for removal – reducing excess nitrogen.
Figure 3.2. Overview of Glucose/Alanine and Alanine Aminotransferase (ALT) Metabolism.

1. In the liver alanine is used in the gluconeogenesis reaction to produce glucose which is then either stored as glycogen or enters the bloodstream to maintain blood glucose levels.

2. As muscle protein is degraded, amino acids are released, which may be metabolised and will release ammonia that is converted to glutamate and free alanine by the ALT reaction. Alanine and glutamine are released into the circulation to return to the liver to release ammonia by the ALT reaction with the ammonia being used to form amino acids or urea. The net result is the removal of ammonia and excess nitrogen from muscle. Alterations in the ALT reaction and its precursors and/or products may indicate alterations in amino acid oxidation in either liver or muscle.

Figure 3.3 shows the detail of the actions that take place in the liver cell mitochondria and cytoplasm for the disposal of the excess nitrogen and ammonia. The relationship between the mitochondrial activity and urea cycle function is shown and involves both ALT and aspartate aminotransferase (AST) activity.
Figure 3.3. Overview of mitochondrial and cytoplasmic urea cycle reactions for ammonia removal.

Figure 3.4. Summary of the metabolism of the oxidative positions of the various amino acids for provision of energy within the citric acid cycle and glycolysis.
Figure 3.4 shows the positions in the citric acid cycle and glycolytic pathways at which catabolism of the various amino acids provide intermediates for energy production. Garber et al, (1976) showed that both alanine and glutamine release from muscle occurred following administration of cysteine, leucine, valine, methionine and isoleucine, whilst increased alanine release alone occurred with administration of aspartate, serine, threonine and glycine. Increases in glutamine alone occurred with administration of tyrosine, phenylalanine and lysine. Therefore differences in the availability of aspartate, serine, threonine, glycine, tyrosine, phenylalanine and lysine will be reflected in differences in blood alanine and glutamine levels. Those amino acids which provide increases in alanine levels feed intermediates into the production of pyruvate and oxaloacetate, whilst glutamine levels are influenced by those amino acids that provide intermediates as acetoacetate or fumarate.

At rest, 6 amino acids are metabolised in muscle (leucine, isoleucine, valine, asparagine, aspartate and glutamate), and provide the ammonia to synthesise alanine and glutamine (Wagenmakers, 1998). The ALT reaction functions to maintain the citric acid cycle intermediates, particularly during the first 10 minutes of exercise and once glycogen is depleted, leucine is oxidised to acetyl-CoA to provide energy intermediates. Exercise-associated muscle fatigue occurs when both glucose and leucine are reduced (Wagenmakers, 1998). Following initiation of exercise, citric acid cycle intermediates are increased, particularly succinate, malate and fumarate, which constitute >90% of the increased citric acid cycle intermediates (Gibala et al, 1997). This increase in intermediate products is associated with increases in alanine and pyruvate and a reduction in glutamine, indicating a primarily glycolysis/ALT-based energy provision and not the ammonia/amino acid-based glutamate dehydrogenase reaction. Thus the ALT reaction along with alterations in citric acid cycle components in the last third of the citric acid cycle (succinate to oxaloacetate) and the amino acids (asparagine, aspartate, tyrosine, phenylalanine) that are oxidised into that part of the cycle, are likely to be good indicators of glycolytic-based fatigue mechanisms. The components of the last third of the citric acid cycle are also those which constitute part of the argininosuccinate-aspartate shunt of the citric acid cycle that links the citric acid and urea cycles.

Figure 3.5 shows the argininosuccinate-aspartate shunt of the citric acid cycle and its association with mitochondrial components, the citric acid and urea cycles, and nitric oxide production. Figure 3.5 shows the importance of the increase in fumarate and malate seen in early exercise activity (Gibala et al, 1997) for the appropriate activity in both the citric acid and urea cycles. The increase in the urea cycle is associated with an increased potential for
the production of nitric oxide (Albina & Mateo, 1995). Nitric oxide is a second messenger molecule which is increased during inflammatory events and if produced in increased amounts for long periods may induce tissue damage (Albina & Mateo, 1995). Thus the ALT reaction along with the amino acids involved in the argininosuccinate-aspartate shunt and its associated citric and urea cycles (succinate, malate, oxaloacetate, asparagine, aspartate, tyrosine, phenylalanine), are likely to not only be good indicators of glycolytic-based fatigue mechanisms, but also those associated with nitric oxide production. If there is loss of precursors for nitric oxide production, the ability to mount an appropriate response may be faulty, which in turn may lead to alteration in host metabolism.

![Figure 3.5. Nitric oxide production, the urea cycle and argininosuccinate-aspartate shunt of the citric acid cycle.](image)

The provision of arginine for the production of nitric oxide and the removal of citrilline after nitric oxide production are important events controlled by the enzymes of the urea cycle. These events are also intricately involved with the activity of the citric acid cycle by the argininosuccinate-aspartate shunt. Fumarate, malate and oxaloacetate are all from the last third of the citric acid cycle. Fumarate, malate and succinate are the components that are increased during the initial stages of exercise. The amino acids that are catabolized to provide energy to this section of the citric acid cycle are aspartate, asparagine, phenylalanine and tyrosine.
It has been proposed that increased tissue damage occurs with prolonged exercise as the latter is associated with increased plasma levels of ALT, AST, creatine kinase, leukocyte counts and cytokine levels (Kayashima et al, 1995; Mena et al, 1996; Bruunsgaard et al, 1997). Chronic strenuous exercise over a 13 week military training program resulted in increases in the white cell count which positively correlated with increases in AST, ALT, lactate dehydrogenase, urea, creatine kinase, superoxide dismutase and uric acid. This suggested that combined muscle and liver damage was occurring along with upregulation of the urea cycle (Kayashima et al, 1995). Bruunsgaard et al (1997) found an increase in interleukin-6 (IL-6), lymphocyte numbers (natural killer cells, CD8 suppressor t-cells) and creatine kinase levels after “eccentric” exercise (contraction with limitation of limb movement), which causes more profound muscle tissue damage, but not normal or “concentric” exercise (contraction with normal limb movement). Conversely, the number of neutrophils increased after concentric exercise but not eccentric exercise. Bruunsgard et al (1997) concluded that an alteration in plasma catecholamines appeared more important in leukocyte recruitment and that the increased tissue damage seen with eccentric exercise was associated with the alterations in the different types of leukocytes recruited between the two forms of exercise. Thus chronic exercise is associated with variable alterations in immune cell numbers and characteristics, which in turn are associated with increased tissue damage (ALT, AST and urea cycle components). TMD patients require investigation to assess whether immune cell and tissue damage occurs with chronic TMD and/or jaw muscle pain. Vega et al (1998) showed that acute injury to limb tissue, using tourniquet application, resulted in increases in serum ALT and AST, as well as nitrate, which is a marker of increased nitric oxide production. Inhibition of nitric oxide production resulted in further increases in serum ALT and AST, whilst enhancement of the nitric oxide response resulted in reduced ALT and AST. They concluded that endogenous nitric oxide production protected the liver from oxidative stress in acute tourniquet shock. As with prolonged exercise (Kayashima et al, 1995; Mena et al, 1996; Bruunsgaard et al, 1997), neutrophil levels increased following transient liver ischaemia-reperfusion (Liu et al, 1998). Inhibition of the nitric oxide response was associated with an increased neutrophil accumulation, superoxide production and liver damage (Liu et al, 1998), which was mediated by neutrophil associated mechanisms independent of nitric oxide production (Wagner et al, 1996). Thus a blunted or inhibited nitric oxide response associated with increased neutrophil superoxide concentrations was important in the increase in tissue damage in transient liver ischaemia-reperfusion (Liu et al, 1995). This is consistent with prolonged exercise-associated tissue
changes (Kayashima et al, 1995; Mena et al, 1996; Bruunsgaard et al, 1997). Other immune mediation factors, such as histamine, may also increase neutrophil adhesion and tissue damage by activation of histamine H1 and H2 receptors but not H3 receptors (Schaefer et al, 1998).

In chapter 2 TMD symptoms were found to have a high association with infectious events and not to be associated with post-exercise fatigue. Similarly the pilot study (Chapter 1) suggested an associated between staphylococcal toxicity and TMD symptoms. Liaudet et al (1997) and Vos et al (1997) both reported that bacterial toxins induce toxic shock as well as tissue and liver damage associated with increases in ALT, AST and nitric oxide production. Toxic shock results from an increase in the production of nitric oxide from L-arginine, and toxin-associated tissue damage (increased serum ALT, AST, urea, creatinine, and lactic acidaemia) and reduced vascular tone (hypotension). Interestingly the amino acid, L-lysine, inhibits L-arginine uptake through the shared amino acid transporter (system y+) and inhibits lactic acidaemia and reduces the organ injury found with endotoxin (Liaudet et al, 1997). It appears that bacterial toxin and cytokine driven changes to urea cycle activity may induce the same changes as noted with prolonged exercise or ischaemia-reperfusion. This suggests that alteration in urea cycle and its associated argininosuccinate-aspartate shunt may play a role in regulation of nitric oxide production and toxicity reactions. Thus the pilot study observation of an association between staphylococcal toxicity and TMD (chapter 1), and the evidence of the association between infectious events and TMD (chapter 2) support an alteration in amino acid metabolism associated with the urea cycle, argininosuccinate-aspartate shunt and citric acid cycle.

Many of the symptoms of CFS patients have been attributed to increased immune cell activation resulting in dysregulated cytokine excretion (sIL-2r, IL-6, Tumour necrosis factor -TNF) (Buchwald et al, 1997). Thus the alterations in leukocytes noted by Bruunsgaard et al (1997), Kayashima et al (1995) and Mena et al (1996) may produce cytokines or other inflammatory mediating substances, which are associated with symptom expression in prolonged exercise. Bruunsgaard et al (1997) reported that increases in lymphocytes and IL-6 levels occurred with eccentric exercise whilst concentric exercise was associated with neutrophil levels suggesting that different inflammatory cytokine increases may not only be associated with muscle pain but may also occur as a result of the condition. Thus assessment of cytokine activity in chronic TMD patients is warranted.
3.2.2. An Overview of Nitric Oxide, Amino Acid Metabolism and Pain.

Nitric oxide is produced from a reaction converting arginine to citrilline (two components of the urea cycle) by the enzyme nitric oxide synthase. (Also known as NADPH Diaphorase; E.C.1.14.13.39).

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\begin{align*}
\text{Arginine} & \rightarrow \text{O}_2 + \text{NADPH} + \text{NADP}^+ \rightarrow \text{NADPH} + \text{NO} \rightarrow \text{Citrilline}
\end{align*}
\]

*Nitric oxide synthase*

Two types of nitric oxide synthase exist: 1) constitutive or continually present in tissues (Nitric oxide synthase 1 in neurones; Nitric oxide synthase 3 in endothelial cells); and 2) inducible (Nitric oxide synthase 2a, induced by endotoxins and cytokines). Both types require \( \text{O}_2 \), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), nicotinamide adenine dinucleotide phosphate (NADPH) and tetrahydrobiopterin as cofactors. Activation of the constitutive types are calcium and calmodulin dependent (reviewed in Albina & Mateo, 1995), whilst the inducible form is found in leukocytes, astrocytes, hepatocytes, endothelial cells and fibroblasts.

Figure 3.5 shows the relationship between nitric oxide production by nitric oxide synthase and the urea cycle. Once produced nitric oxide is rapidly converted to the stable compounds \( \text{NO}_2^- \) and \( \text{NO}_3^- \) (Albina & Mateo, 1995). The type of reactive metabolite produced with activation of nitric oxide production is determined by the redox potential of the cell in which it is produced (Lipton et al, 1993) and has the paradoxical outcome of being either protective or toxic. The active by-products of nitric oxide formation react with atoms or molecules that contain unpaired electrons, such as oxygen, superoxide and metal ions (iron, copper, and manganese). Various active nitric oxide by-products combine with iron in haem proteins, such as haemoglobin, with the resultant production of the second messenger molecule, cyclic-Guanosine 5’-monophosphate (c-GMP). Nitric oxide by-products can inactivate enzymes with iron-sulphur prosthetic groups and tyrosine residues. Target enzymes and molecules include the citric acid cycle and mitochondrial electron transport associated enzymes, aconitase, ubiquinone and succinate oxidoreductases as well as ferritin, ribonucleotide reductase, AMP deaminase, and the glycolysis/gluconeogenesis enzyme glyceraldehyde 3-phosphate dehydrogenase (Albina & Mateo, 1995). Thus nitric oxide has profound effects upon many enzyme systems and molecules and is associated with many physiological changes including neural, cardiovascular, pulmonary, renal, gastrointestinal
and endocrine functions (reviewed in Albina & Mateo, 1995), and may have protective and destructive actions.

Pain responses in both the central nervous system (Coderre, 1993; Coderre & Yashpal, 1994; Wong et al, 1998) and peripheral tissues (Holthusen & Arndt, 1995; Kang et al, 1995; Lorenzetti & Ferreira 1996) are associated with increased nitric oxide production. Coderre & Yashpal (1994) showed that activation of receptors or cellular mechanisms that increase the intracellular messengers, nitric oxide, arachidonic acid and protein kinase C lead to persistent nociception in response to tissue injury in rats. Central sensitisation induced by excitatory amino acids and substance P was reversed by pre-treatment with nitric oxide, arachidonic acid and/or protein kinase C inhibitors. These nitric oxide-related changes are linked with a decrease in the detection and tolerance thresholds of pain in humans (Thomsen et al, 1996) and may be initiated by a number nitric oxide radical generating mechanisms (Machelska et al, 1997; Aley et al, 1988). The primary factor associated with this alteration in the hyperalgesic pain threshold may be the N-Methyl-D-Aspartate (NMDA)-associated inhibition of Na⁺K⁺ATPase activity (Tavalin et al, 1997). These receptor associated events mediate a process called excitotoxicity (Thomas 1995) which in the nervous system is commonly associated with increased action of excitatory amino acids, glutamic and aspartic acids, on NMDA and similar receptors. Hyperalgesia mediated by NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors is linked with nitric oxide accumulated in a population of small cells and neuropil (an area of intermingled neuronal processes associated with synaptic junctions) in laminae II and III of the dorsal horn, consistent with a role in the processing of nociceptive signals (Morris et al 1994; Hama & Sagen, 1994). Interestingly, Siegan et al (1996) showed that implanting adrenal medullary cells into the spinal cord subarachnoid space resulted in reduction of hyperalgesia and allodynia, suggesting that alterations in opoids and catecholamines (dopamine, noradrenaline, adrenaline) may modulate the spinal nitric oxide associated pain mechanisms. Altered nitric oxide production in the central nervous system has been suggested to be involved in phantom limb pain (Neidbala et al, 1995) and neuropsychiatric disorders (Karatinos et al, 1995). Apart from peripheral nociceptive activation, bacterial lipopolysaccharide (LPS) (Wiertelak et al, 1994a, Watkins et al, 1994) and interferon-γ (IFN-γ) (Xu et al, 1994) associated with inflammatory events can also activate the spinal column NMDA-nitric oxide cascade and induce hyperalgesia. Watkins et al (1994) also showed that substance P, cholecystokinin and excitatory amino acids acting at sites other than NMDA, serotonin or kappa opiate receptors,
mediate this bacterial LPS associated spinal hyperalgesia. Thus nitric oxide, excitatory amino acids and catecholamines have a complex role in the modulation of pain responses in the central nervous system.

Activation of peripheral nociception is also involved in pain mechanisms. Peripheral stimuli such as the herniation of a cervical intervertebral disc (Holthusen & Arndt, 1994) or the injection of nitric oxide into peripheral tissues are associated with increased pain (Kang et al, 1995). Kawabata et al (1994) assessed the peripheral activity of nitric oxide on formalin induced hyperalgesia. Increased arginine levels facilitated the second phase of nociceptive responses at low doses but had antinociceptive activity in higher doses. Conversely, inhibition of nitric oxide production reduced the nociceptive response of the second phase. Thus variation in nitric oxide activity modulates peripheral nociceptive activity with reduced levels or blunted responses leading to enhanced nociceptive activity. These activities are separate from the central nervous system activities (Wiertelak et al 1994b) and the same pain associated modulatory actions of nitric oxide concentration are not seen in the spinal cord (Malmberg & Yaksh, 1993). Also of significance in peripheral tissues are the roles nitric oxide scavengers such as haemoglobin and amino acids, which by binding nitric oxide radicals modulate nitric oxide activity or availability (Inoue et al, 1998). Thus in peripheral tissue the levels of nitric oxide and the availability of nitric oxide scavengers, such as haemoglobin, may modulate the pain response in a different manner to that seen in the central nervous system.

Lipton et al, (1993) found that nitric oxide activation in neural tissue can result in the paradoxical outcome of either neuroprotective or neurotoxic activity. This change in activity was associated with the redox-state of the neurone. Activation of NMDA receptors along with nitric oxide production and superoxide anion presence lead to the formation of peroxynitrate (ONOO⁻) and neurotoxicity. Conversely, down-regulation of NMDA receptors along with nitric oxide production and the formation of NO⁺ are neuroprotective. These events were regulated by the redox status of the cell and have also been shown to have similar protective and toxic activities in bacterial cells (De Groote et al, 1995). Interestingly, the prolonged activation of the nitric oxide response is associated with post-transcriptional modulation of nitric oxide synthase activity (Zhang et al, 1998), which was associated with an 80% decrease in the redox control protein, thioredoxin, and a 27% decrease in thioredoxin mRNA expression. Thioredoxin is a redox control protein that is produced in response to cellular redox stressors and is an important intracellular redox control system (reviewed in Nakamura et al, 1997). Thus nitric oxide is able to modulate the redox-state of cells by
changes in redox protein levels, which in turn may result in differences in the cellular production of nitric oxide by-products and quite different molecular outcomes.

Nitric oxide production, the redox status and Na⁺K⁺ATPase function may alter neuronal excitotoxicity and pain responses. Sato et al (1997) found that the inhibition of Na⁺K⁺ATPase by nitric oxide was associated with the oxidation state of the cell with sulphur reducing agents (reduced glutathione, cysteine) or oxide scavenging agents (ascorbic acid) able to inhibit the effect of nitric oxide. Once Na⁺K⁺ATPase was inhibited by nitric oxide only the SH reducing agents were able to reverse the inhibition. The production of different nitric oxide radicals was associated with different patterns of Na⁺K⁺ATPase inhibition. Superoxide associated peroxynitrate induced Na⁺K⁺ATPase inhibition but was attenuated by nitrogen dioxide whilst nitrogen dioxide in the absence of superoxide induced Na⁺K⁺ATPase inhibition. Thus nitrogen dioxide had both agonist and antagonist effects on Na⁺K⁺ATPase activity which was dependent upon the presence of other Na⁺K⁺ATPase active metabolites. Boldyrev et al (1997) showed that nitric oxide radicals combined with iron complexes were the most potent inhibitors of Na⁺K⁺ATPase. Restoration of the sulphur groups on the Na⁺K⁺ATPase using cysteine restored the activity. Thus the activity of Na⁺K⁺ATPase is significantly influenced by nitric oxide radicals and the redox potential of the cell and may play a significant role in the pain associated hyperalgesic response.

Other factors have been found that may alter nitric oxide production and the outcome of the normal response. A combined increase in bacterial LPS and the cytokine IFN-γ, which are inducers of Nitric oxide synthase 2A, can modulate nitric oxide production and also the redox status of the tissues (Hecker et al, 1996; Hothersall et al, 1997). Similarly, a combined exposure of superoxide and interleukin-1β (IL-1β) resulted in a 10-fold amplification of nitric oxide production (Beck et al, 1998). Grossie et al (1997) have reported that patients with tumours have an enhanced toxicity to bacterial toxins, and that bacterial endotoxaemia was significantly increased in rats with transplanted Ward colon tumours compared with control rats. In the tumour burdened rats, the nitric oxide response was greater and there was an increase in serum ALT levels along with an increase in ornithine levels, a component of the urea cycle that accumulates with increased nitric oxide production (Albina et al, 1990). Thus bacterial toxins, inducers of nitric oxide synthase 2A, appear to be able to induce enhanced toxicity in disease states where nitric oxide is already being produced by other stimuli, such as tumours and inflammatory events. Interestingly inhibition of the nitric oxide response results in attenuation of bacterial-associated toxaemic shock (Ruetten et al 1996).
Thus alterations associated with the redox potential and the ability to induce nitric oxide responses, appear to play a significant role in pain responses and may be enhanced by secondary bacterial toxicity.

### 3.2.3. Organochlorine Pesticides in Polysymptomatic Pain/Fatigue Patients.

Chlorinated hydrocarbons such as hexachlorobenzene (HCB) and dichlorodiphenyltrichloroethane (DDT, or its metabolite 1,1-dichloro-2,2-bis(p-chlorophenyl)ethane, DDE) are recalcitrant chemicals, which have been used extensively world wide for pest control since the early 1940’s and have been reported to have oestrogen-like and anti-anabolic effects. A cohort of subjects with defined chronic fatigue syndrome had an increased incidence and mean concentration of total serum chlorinated hydrocarbons compared with age- and sex-matched controls (Dunstan et al, 1995). Dunstan et al (1996) reported that patients with high DDE levels had a reduced percentage lymphocytes and an increased number of neutrophils and total white cell count (Dunstan et al, 1996) suggesting that organochlorines such as DDE may predispose the host to increased infections. Dunstan et al (1995) found DDE in 100% of subjects tested which is consistent with the observations of Stellman et al (1998) that found DDE in all adipose and serum samples taken from humans with the adipose tissues having much greater tissue levels. Stellman et al (1998) also found that serum and adipose tissue DDE levels were significantly correlated and they concluded that either serum or adipose tissue levels can be used as a measure of the body burden of the pesticide. Kreiss et al (1981) found that the total DDT and DDE levels were not associated with any specific illness or ill health, however, total DDT and DDE levels were positively associated with levels of serum cholesterol, triglyceride, and gamma-glutamyl transpeptidase (Kreiss et al, 1981). Ando & Wakisaka (1975) have previously reported the positive association between retained DDE levels and triglyceride levels in the serum and liver.

DDE is an anti-androgenic compound as it inhibits androgen binding to the androgen receptor (Kelce et al, 1995; Kelce et al, 1997; Sohoni & Sumpter, 1998). DDE was also found to inhibit Na⁺K⁺ATPase activity and glucose and L-tyrosine transport in rats (Iturri et al, 1989; Iturri & Wolff, 1982). Interestingly DDE has also been associated with a reduction in dopamine and noradrenaline levels in doves (Heinz et al, 1980), a reduction in brain γ-aminobutyric acid (GABA) and kidney tyrosine, proline and valine suggesting a sodium mediated alteration in amino acid transport (Dikshith et al, 1975). Also of importance, Ferreira et al (1997) reported that DDE interacted with mitochondrial complex II (succinate
dehydrogenase) and results in a reduction in oxidative phosphorylation and membrane potential. These data suggest that organochlorine especially DDT and DDE may alter fatigue and the nitric oxide associated pain mechanisms.

Another organochlorine pesticide, Dieldrin, binds to both gamma-aminobutyric acid (GABA), the major inhibitory amino acid in the central nervous system, and monoamine receptors (Brannen et al, 1998; Sanchez-Ramos et al, 1998). Alterations in the GABA and monoamine receptors were most prominent in the brain stem, where the trigeminal nerve nuclei are found (Brannen et al, 1998). These data suggest that Dieldrin may also have effects upon TMD symptom expression.

Due to the potential oestrogen-like, anti-anabolic influences and the induced changes in neurotransmitter activity of organochlorine pesticides and the increased female: male ratio for TMD an examination of the associations between organochlorine pesticides and TMD is warranted.

3.2.4. Alteration of Amino Acids in Polysymptomatic Pain/Fatigue Patients.

Total serum amino acid levels, particularly proline, serine, tryptophan and histidine were reduced in a cohort of patients with fibromyalgia (Russel et al, 1989), whilst reductions in serine and histidine and a potential anomaly in tryptophan transport have also been reported (Yunus et al, 1991). Patients with CFS have alterations in urinary excretion of amino and organic acids (McGregor et al, 1996a, 1996b, 1996c). Unknown molecule CFSUM1, together with tyrosine, β-alanine, aconitic acid and succinic acid were increased, whilst serine, alanine and glutamic acid were reduced compared with control subjects. These changes in urinary excretion patterns suggest an alteration in biochemical homeostasis involving enhanced protein degradation or proteolysis in patients with CFS. Furthermore, CFS patients with increased pain severity on a visual analogue scale had increases in urinary excretion of aspartic acid and CFSUM1, and reductions in serine and phenylacetic acid. Aspartic acid is a product of the AST reaction, but is also an excitatory amino acid that can activate the NMDA receptor associated hyperalgesic response as has been described in chronic pain (Coderre, 1993; Coderre & Yashpal, 1994; Thomas 1995). Additionally aspartic acid is a major amino acid substrate for the formation of argininosuccinate and arginine, the nitric oxide precursor (Figure 3.5) suggesting that increases in aspartic acid may enhanced the bodies ability to produce nitric oxide. These data provided evidence for a potential mechanism describing chronic pain in vivo, involving dysregulation of pain modulating
neurotransmission and increased levels of excitatory amino acids leading to persistent nociceptive responses.

In fibromyalgia and CFS patients, the basal excretion of urinary catecholamines (Yunus et al, 1993) and the levels of hypothalamic-pituitary-adrenal axis neuropeptides were no different from controls (Cleare et al, 1995). However, patients with fibromyalgia and CFS had altered hypothalamic-pituitary hormonal excretion responses. Whilst fibromyalgia and CFS have been linked to depressive illness, the altered central nervous system (CNS) hormonal responses in patients with major depression, are distinct from those seen in CFS and fibromyalgia patients (Cleare et al, 1995; Moeller et al, 1994). It is now well accepted that CFS and fibromyalgia patients have dysregulation of prolactin and growth hormone excretion whilst major depressive patients have a dysregulated cortisol excretion (Cleare et al, 1995). Aguilar et al (1997) showed that prolactin secretion from the pituitary was associated with activation of NMDA receptor/nitric oxide/dopamine related mechanisms, which could also be inhibited by testosterone. Increased excitatory amino acid and nitric oxide levels, and reduced dopamine levels result in increased prolactin release. McCann et al (1998) discussed the evidence for alterations in the control of these hypothalamic/pituitary excretory mechanisms and its association with bacterial toxins, cytokines and nitric oxide production. The dysregulated prolactin/growth hormone secretion seen in chronic pain patients, is highly suggestive of a nitric oxide-dependent combined bacterial LPS/interleukin-1α,β (IL-1α, IL-1β) mediated mechanism, associated with a reduction in dopamine release (McCann et al, 1998). This is supported by the observation in fibromyalgia patients of reductions in serum cortisol, adrenaline and noradrenaline (Van Denderen et al, 1992).

No studies on TMD patients have been undertaken into these mechanisms, however data associating TMD with behavioural changes such as depression (Buckelew et al, 1986; Clark et al, 1985; Lee & Lee 1989; Pelz & Merskey 1982; Von Korff et al, 1988; Vimpari et al, 1995) have resulted in the psychophysiological hypothesis for TMD. Similarly TMD symptoms are associated with prescribed drugs that dysregulate dopamine metabolism, which also support this possibility, and is reviewed in Chapter 2 (Lynch et al, 1961; Malkin, 1964; Evans, 1965; Hiatt & Schwartz, 1966; Kraak, 1967; Pertoutka et al, 1988). In addition use of tricyclic anti-depressants (desipramine, nortriptyline, imipramine) that increase central nervous system noradrenaline availability and activity, have been used for pain relief in TMD patients (Tura & Tura, 1990). These data suggest that a reduction in noradrenaline and/or an increase in nitric oxide are likely to influence pain expression in TMD patients. The possible
association between nitric oxide increases and TMD has been proposed by Anbar and Gatt (1988), and evidence of a possible increased nitric oxide response in TMD patients is provided by the reduction in mean blood pressure (de Abreu et al, 1993). Thus evaluation of amino acid metabolism in TMD patients is warranted.

3.2.5. Orofacial Pain Mechanisms.

Chronic pain phenomena are associated with NMDA associated hyperalgesia in both the CNS (Coderre, 1993; Coderre & Yashpal, 1994; Wong et al, 1998) and peripheral tissues (Holthusen & Arndt, 1995; Kang et al, 1995; Lorenzetti & Ferreira 1996). Coderre & Yashpal (1994) showed that activation of receptors or cellular mechanisms that increase the intracellular messengers, nitric oxide, arachidonic acid and protein kinase C lead to persistent nociception in response to tissue injury in rats. Hu et al (1993) and Yu et al (1995) revealed that jaw and neck muscle hyperactivity could be influenced by injection of mustard oil. Broton & Sessle (1988) and Broton et al (1988) showed that other algesic substances such as NaCl, KCl, bradykinins and histamine also activate the hyperalgesic process in the trigeminal system. Chiang et al (1997) showed that NMDA receptor activation was involved in the trigeminal nociceptive/hyperalgesic mechanism and that inhibition of the total receptor and the glycine receptor site inhibited the algesic response. Yu et al (1996) had previously shown that the NMDA receptor was involved in the development of increased jaw muscle electromyographic activity. Watanabe et al (1999) showed that antihistamines had an inhibitory effect upon palpable muscle tenderness in the head, neck and face. Thus alterations in nitric oxide, sodium, potassium and inflammatory mediators such as histamine may be involved in trigeminal nerve hyperalgesic responses, increased jaw muscle activity and pain.

3.2.6. Proteolysis (Intracellular Protein degradation).

The alterations in amino and organic acids in fibromyalgia and CFS patients may be the result of dysregulation of cellular protein turnover where there is a persistent increase in proteolysis over protein synthesis. This enhanced proteolysis is a mechanism that allows increased provision of amino acids from both muscle and liver following trauma, infection or highly stressful situations (Mortimore & Poso, 1987; Hasselgren, 1995). This mechanism has also been described in patients with certain genetic and acquired disease states, in muscle wasting associated with late stage cancer, as well as with cytokine action (Mortimore & Poso, 1987; Hasselgren, 1995).
Muscle proteolysis consists of two major mechanisms, the removal of: 1) short-term proteins (cytoplasmic proteins) or non-fibrillar proteolysis; and 2) long-term proteins (contractile proteins) or fibrillar proteolysis. In muscle, enhanced proteolysis can be related to both non-fibrillar (short-term proteins) and fibrillar (long-term protein - actin, myosin) mechanisms. Non-fibrillar proteolysis is associated with increased release of tyrosine from the cytoplasmic protein pool, whilst fibrillar proteolysis is associated with increased release of the unique actin component, 3-methylhistidine from muscle fibres (Hasselgren, 1995; Mortimore & Poso, 1987). Thus for muscle, these two processes can be assessed, using the release of tyrosine (non-fibrillar) and 3-methylhistidine (fibrillar), similar to that used to assess the actions of cytokines in proteolysis (Hasselgren, 1995). However the liver is also a major site of release of amino acids during sepsis, trauma and stressful events (Haussinger & vom Dahl, 1995). Liver and muscle, act as major control and storage sites for the release of amino acids during proteolytic events, and have different responses to cortisone, glucagon and noradrenaline (Hasselgren, 1995; Haussinger & vom Dahl, 1995). In sepsis, there is a generalised reduction in muscle amino acids as a result of reduced uptake of amino acids and increased efflux of amino acids from muscle tissue (Roth et al, 1982; Austgen et al, 1992). Conversely, in the liver, amino acid uptake is increased during sepsis (Ardawi, 1992; Austgen et al, 1991) and can be stimulated by both glucagon and noradrenaline (Canivet et al, 1980).

Muscle fibrillar and non-fibrillar proteolysis are two separate and independent processes (Mortimore & Poso, 1987). Muscle fibrillar proteolysis requires activation of calcium dependent proteases; whilst the non-fibrillar proteolytic response is controlled by insulin, glucagon, growth hormone, variation in cell volume, cyclic AMP, heavy metals, cytokines, the redox potential as well as the variation in specific amino acids (Tischler 1980; Rinehart et al, 1982; Fagan et al, 1987; Mortimore & Poso, 1987; Haussinger et al, 1991; Jimenez Jimenez et al, 1991; Balavoine et al, 1993; Fryburg & Barrett, 1993; Simmons et al, 1994; Hasselgren, 1995). Control of proteolysis in the liver is associated with variations in amino acids such as leucine, tyrosine, phenylalanine, glutamine, proline, methionine, tryptophan and histidine as well as an unusual promotional effect from alanine (Haussinger & vom Dahl, 1995). At normal physiological levels, the inhibitory effects of leucine and the other amino acids on non-fibrillar proteolysis, are potentiated by alanine (Mortimore & Poso, 1987). The increase in alanine levels during early exercise is thus likely to inhibit proteolysis and enhance protein synthesis in the liver (Wagenmakers, 1998). The amino acid leucine controls non-fibrillar proteolysis in muscle and adipose tissue, and has a distinct influence
over RNA degradation (Balavoine et al., 1993). Thus alterations in the homeostasis of the proteolysis controlling amino acids may potentiate a dysregulation of the control of non-fibrillar proteolysis and may also reflect differences in muscle and liver amino acid metabolism. The variation in the pattern of urinary and serum amino acids are likely to reflect the underlying mechanisms associated with proteolysis and symptom expression.

Proteolysis and protein synthesis are closely associated with alterations in the redox potential and the ratio of oxidised nicotinamide adenine dinucleotide (NAD\(^+\)) and reduced nicotinamide adenine dinucleotide (NADH) (Tischler, 1980; Rinehart et al., 1982; Fagan et al., 1987). Fagan et al. (1987) showed that the induction of diabetes resulted in an alteration in the NADH: NAD\(^+\) ratio and proteolysis, and suggested that alterations in insulin levels may play a major role in regulation of these processes. Hedden & Buse (1982) showed that whilst most NADH is derived from glycolysis, that degradation of the branched chain amino acids, leucine and isoleucine, will also result in increased NADH production and alteration in the NADH: NAD\(^+\) ratio. Similarly the other proteolysis/protein synthesis associated hormones (glucagon, prolactin, growth hormone) also alter the NADH: NAD\(^+\) ratio (Sistare & Haynes, 1985) and are associated with altered uric acid excretion (Yamamoto et al., 1996). These data indicate that the control of proteolysis exerted by leucine and the hormones such as insulin and glucagon, may be the result of alteration in the NADH: NAD\(^+\) ratio. It is therefore worthy to note that Oles (1977) reported that anomalous glucose tolerance was associated with TMD symptoms; and Aaflot & Bruunsgaard (1994) identified that increasing levels of uric acid had an increased odds ratio for the development of fibromyalgia.
3.3. METHODS.

3.3.1. RDC\TMD type 1a patients.

3.3.1.a. Patient and Control Selection.

Forty-six patients sequentially presenting for orofacial pain management, complaining of orofacial muscle pain were recruited as previously described in chapter 2. These patients were designated to be the RDC/TMD type 1a patient group. Patients had a positive response on a VAS of average pain intensity in the 2 weeks prior to consultation, the presence of palpable muscle pain in the reported pain areas and that the pain was present on greater than 50% of days during the three months immediately preceding consultation.

Forty-one age and sex matched control subjects were recruited including, relatives of the pain patients and unrelated subjects to ensure similar socio-economic and ethnic backgrounds, as previously described in chapter 2. Control subjects (C), were eligible for inclusion in the study if they had no pain response to a VAS in the 2 weeks prior to consultation, did not give a history of chronic pain and had not sort professional advice or treatment for chronic muscle pain in the previous 12 months as previously described in Chapter 2.

3.3.1.b. Questionnaires.

All subjects completed a CPRU questionnaire and a Hopkins Symptom Check List-90-Revised (SCL-90-R), as previously described in chapter 2. The symptom incidence index was calculated as the number of positive responses to 48 symptoms from within the CPRU as previously reported in chapter 2.

3.3.1.c. Confirmation of Muscle Pain.

Muscle pain was confirmed by palpation of superficial facial, neck and shoulder and muscle pain and symptom severity was assessed using a VAS and the SCL-90-R question responses (Questions 1, Q4, Q12, Q14, Q27, Q39, Q40, Q42, Q52, Q55, Q56, Q58, Q66) as previously described in chapter 2.

3.3.1.d. Urine Specimens and GC-MS Identification.

Each subject collected the first morning urine specimen on the day of the second visit. The urine was refrigerated (not frozen) and processed within twenty-four hours of collection.
10mL aliquots were centrifuged at 1,500g for five minutes at 4°C and 200µL aliquots were transferred to derivatization tubes and freeze dried for eighteen hours.

The freeze dried urine material was then reacted to form the N(O,S)-heptafluorobutyryl-isobutyl (HFB-isobutyl) derivatives for analysis by GC-MS (MacKenzie & Tenaschuk, 1979a; 1979b). Two hundred microlitres of HCL-isobutanol were added to the freeze dried sample and heated at 110 °C for one hour. After cooling and evaporation by high purity nitrogen gas, the sample was freeze dried for thirty minutes. Ethyl acetate (50µL) and heptafluorobutyric anhydride (20 µL, HFBA) were added and the sample heated at 110 °C for thirty minutes. After cooling, the sample was evaporated with nitrogen gas and freezes dried for five minutes and then dissolved in 200µL of ethyl acetate. Derivatized urinary metabolites were separated using a Hewlett Packard 5890 Series II gas chromatograph (GC) and detected by a Hewlett Packard 5971A mass selective detector (MSD) as described previously (Walsh et al, 1995). Quality control was achieved by running a selected sample source every week with each batch of analyses to monitor extraction efficiency and machine response, and quantitative responses were assessed by an external standard strategy. Internal standard strategies were not used because these standards may co-elute with novel disease related metabolites in the chromatogram. Thirty-six urine peaks were selected for examination after confirming a linear detector response by the mass spectrometer. This is an extended list of metabolites from that used in the previous studies (McGregor et al, 1996a). Where possible, the peaks were identified by HP-UX Chemstation computer search of user-generated reference libraries (incorporating retention indices and mass spectra) and the WILEY Database™. The original peaks were numbered one to 28 in order of retention time and those peaks that could not be identified were allocated a reference code (either UM or CFSUM) as previously described (McGregor et al, 1996a). The additional peaks were allocated an alphabetic suffix (e.g., UM13a, UM15a) to conform to the retention time peak allocation method previously described (McGregor et al, 1996a). The area of each peak was recorded and percentage abundance of the 36 peaks was calculated for further analysis. The relative abundance analysis was included to allow assessment of metabolite changes, which were independent of variation in urine volume and total metabolite excretion.

Relative abundance or qualitative analyses of urine metabolites were performed using percentage analyses as used in previous studies (McGregor et al, 1996a; 1996b, 1996c). Quantitative urine analyses usually involve obtaining 24-hour urine samples. However, this study aimed to minimise dietary influence and standardise collection procedures, as well as
maintain consistency with previous investigations, which have shown the value of this approach (McGregor et al, 1996a; 1996b, 1996c). The data were reviewed to assess possible quantitative differences and associations, which may be apparent from these types of urine analyses. Since 10 of the 36 measured urinary metabolites were unidentified components, the data were processed as GC-MS arbitrary peak area response units/µL, for statistical evaluation of group differences and associations.

3.3.2. CFS Group.

3.3.2.a. Study Population.

These data were collected as part of a study on CFS patients and not specifically directed at the TMD symptoms. This thesis reports on the association between reported TMD symptoms and biochemistry within this CFS population.

CFS patients and age and sex matched control subjects were recruited over an 18-month period, September 1994 - February 1996. The calculated study group sizes were required to be a minimum of 80 subjects per arm. The CFS patients were recruited from sequential medical practitioner referrals to the CFS clinical group at Royal North Shore Hospital from within the Sydney metropolitan area, and from advertisement in an ME/CFS newsletter. Two hundred and ten potential CFS subjects expressed an interest in participating in the study, of whom 114 agreed to participate and met the Centers for Disease Control (CDC) criteria (Holmes et al, 1988). Defined CFS patients were excluded at any time if they were given any alternative diagnosis or did not comply with additional study requirements.

The age- and sex-matched control subjects were recruited from two sources; friends and relatives of the CFS patients; and via advertisement of project patient recruitment at the University of Sydney and the University of Technology (Sydney). These two groups were selected to ascertain any differences between control subjects with exposure to CFS patients and control subjects with no exposure to CFS patients. One hundred and eight control subjects were assessed and excluded if they reported fatigue that influenced their lives or had evidence of any diagnosable illness or psychiatric disorder. Eighty-six control subjects were recruited.

All study participants were requested to cease the use of any alternative medicine or vitamin supplement for one week prior to sample collection. On the day of clinical examination and sample collection subjects were clinically assessed by either of 2 clinicians (PC-B, GF), completed specifically designed questionnaires, the John Hopkins Symptom
Checklist-90-Revised (SCL-90-R) (Degrotis, 1975) and had blood, microbiological swabs of nose and throat, venous blood and urine samples collected. The specifically designed questionnaires addressed medical history, symptom prevalence and severity, onset and duration of illness, treatments used, social demographics and other factors which may influence their illness (Appendix).

TMD symptoms were assessed clinically as presence or absence of facial pain and from scalar responses in a questionnaire to 12-month frequency and severity and 7 day severity to facial pain and TMJ pain. The questionnaire assessed the scalar severity response to facial and TMJ pain within the last 7 days and the last 12 months as well as a scalar response of the frequency of facial or TMJ pain over the last 12 months. No TMD clinical signs could be elicited in this study group as the study design explicitly excluded the author from any clinical involvement to ensure objectivity.

3.3.2.b. Urine Specimens and GCMS Compound Identification.

The collection of urine varied from that in the RDC/TMD type 1a patients. Each study subject recorded the time of the last urination during the evening prior to the day of sampling. All urine voided from the last voiding to the following appointment was collected into a 1L sterile container and the time of the last voiding recorded. The urine samples were coded, refrigerated (not frozen) at the collection point and transported to the laboratory for processing within twenty-four hours of collection as described in section 3.3.1.4. The excretion rate per minute was calculated by multiplying the peak area per ml of urine by the number of mls of urine excreted per minute and dividing the total by 1000. Thus 3 different urine data sets were available for analysis (excretion rate per minute, percentage excretion rate and absolute amount excreted) in the CFS cohort compared with 2 in the TMD/RDC type 1a cohort.

3.3.2.c. Blood Sample Collection and Analyses.

A 10mL blood specimen was collected in the early morning from overnight fasted subjects by venepuncture into glass EDTA vacutainers. Blood samples were coded stored at 4⁰C and blood parameters assayed within 24 hours. The standard full blood count and serum chemistry was performed at the Royal North Shore Hospital Pathology Laboratories using a Coulter STKS (Coulter Electronic, Sydney, Australia). Blood samples collected in Becton Dickinson lithium heparin vacutainers, at the Royal North Shore Hospital, were stored at 4⁰C and transported to the University of Newcastle. Samples were stored on ice during
transportation, which occurred within 3-6 hours. On arrival at the University of Newcastle the plasma component of the whole blood samples was separated by centrifugation at 3000 rpm for 25 minutes at 4°C (Beckman GS-15R centrifuge). All samples were processed within 8 hours of collection.

3.3.2.d. Extraction of Amino and Organic acids from Plasma.

Lipophilic components were extracted from the plasma, using methanol, to precipitate proteinaceous material, and a mixture of hexane and diethyl ether. The sample was vortexed for 60 seconds and centrifuged at 3000 rpm for 10 minutes at 4°C. The upper hexane/ether phase was removed by Pasteur pipette and discarded. Following lipid extraction, the sample was again vortexed for 60 seconds and centrifuged at 3000 rpm for 20 minutes at 4°C using methanol extraction. 2mL aliquots were transferred to a clean derivatization tubes and an internal standard (50µL pipecolic acid - 1nmol in distilled water) was added. Samples were snap frozen using liquid nitrogen and freeze-dried overnight, with the remaining alcohol removed by lyophilization.

HFB derivatization of the freeze-dried sample occurred as per section 3.3.1.4. Twenty-five plasma peaks were selected for evaluation. Where possible chromatographic peaks were identified by comparing the mass fragmentation patterns of each component together with its retention index to those held in mass spectral data libraries (user-generated reference libraries incorporating retention indices and mass spectra and the WILEY Database™) by HP-UX Chemstation computer search. Those peaks that could not be identified were allocated a reference code, unknown ph1 to unknown ph4. The data were expressed as mean peak area per mL of plasma. Standard curves for the amino acids ethanolamine, serine, alanine, glycine, β-alanine, valine, threonine, leucine, isoleucine, proline, s-methylcysteine, asparagine, aspartic acid, phenylalanine, ornithine, glutamic acid, lysine, tyrosine, aconitic acid and tryptophan were produced.

3.3.2.e. Extraction of Pesticide residues from Plasma.

The pesticide residues were extracted from plasma by the methods described in Dunstan et al (1995). The following pesticides were assessed: Aldrin, α- β- γ-BHC, Clordane, DDT, DDE, Deildrin, Endrin-1, Endrin-2, Endrin aldehyde, Endrin ketones, Endo sulphate, HCB, Heptochlor, Heptochlor epoxide, methoxychlor and PCB. Of these pesticides γ-BHC, Clordane, DDT, Endrin-1, Endrin-2, Endrin aldehyde, Endrin ketones, Endo
sulphate, Heptochlor epoxide, methoxychlor and PCB were not detected in any blood samples from the CFS or control subjects. Therefore the analysis was restricted to Aldrin, α-β-BHC, DDE, Deildrin, HCB and Heptochlor.

3.3.3. RNase-L CFS study.

3.3.3.a. Patient selection.

This data set formed the experimental group in this study for the BSc(Hon) degree by Ms Lee Metcalf with the express purpose of examining the differences between CFS patients and control subjects. The TMD data was not reported and allows analysis of the associations between sIL-2r, IL-6, RNase-L and TMD.

Forty-two CFS patients who complied with the Fukuda et al (1994) CFS definition were recruited from general medical practitioners in the lower Hunter River district, after all subjects had the necessary clinical assessment to exclude all other possible causes of fatigue. Fifty community-based age- and sex-matched healthy control subjects were recruited from the friends and associates of the CFS patients. Thus these control subjects were close contacts of CFS patients. University of Newcastle Ethics approval was obtained. Patients completed a questionnaire that obtained scalar responses (0-4) to 86 questions on physical, cognitive and psychological symptoms over the preceding 7 days. This questionnaire is used as a standard questionnaire for poly-symptomatic patient symptom assessment (Appendix 4).

3.3.3.b. Blood Sample Collection and Analyses.

A 10mL blood specimen was collected in the morning from study subjects (32 CFS and 31 controls) by venepuncture into glass EDTA vacutainers. Blood samples were coded, stored at 4°C and blood parameters transported to a commercial laboratory for assessment of RNase-L activity, and the levels of soluble IL-2 receptor (sIL-2r) and IL-6 (Melbourne Forensic Diagnostic Laboratories, Melbourne, Australia). The RNase-L activity was assessed as total RNase-L and was not assessed to allow distinction between the 37Kd RNase-L identified to be increased in CFS patients (Suhadolnik et al, 1997).

3.3.4. Statistical Analysis.

All percentage data were arcsine transformed before analysis and other data not having a normal distribution were log transformed for usage in logistic regression analysis. Subject characteristics and symptom incidence was assessed using chi-square probability and
students t-tests. Correction for multiplicity occurred where necessary. Univariate group differences were assessed on untransformed data using the non-parametric Mann Whitney U test and Spearman rank correlation, and ANOVA. Group differences were further assessed using multivariate standard and forward-stepwise discriminate function analyses, Pearson product moment analyses on the transformed data (significance $P \leq 0.05$; NS = not statistically significant). These data were processed using Access97™ (Microsoft, Redmond, WA, USA), Excel97™ (Microsoft) and Statistica™ (Ver. 5.1, Statsoft, Tulsa, OK, USA).