MUSCLE DAMAGE AND SORENESS FOLLOWING PROLONGED

INTERMITTENT SHUTTLE RUNNING AND THE EFFECT OF

VITAMIN C SUPPLEMENTATION

by

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ABSTRACT

Exercise-induced muscle soreness and damage have been investigated for almost a century, and yet it appears that there is little that can be done to avoid these consequences of over-exertion, except train on a regular basis. It is likely that free-radicals are involved at a number of stages in the muscle damage process, and therefore the provision of appropriate antioxidants may theoretically offer some protection. One such antioxidant is vitamin C, although the literature available in support of this notion is scarce. The aim of these studies, therefore, was to assess whether different nutritional interventions using vitamin C would offer any benefit to exercise-induced muscle damage and soreness.

In the past, investigators have often used exercise protocols designed to maximise the extent of injury. The studies reported in this thesis, however, used an exercise protocol (Loughborough Intermittent Shuttle Test: LIST) based on the multiple-sprint sports (e.g. football). Participation in such sports is very high, although frequently on an irregular basis, and therefore exercise of this nature may have the capacity to cause muscle damage and soreness. The LIST provided a suitable exercise model, and in different studies led to increases in soreness, markers of muscle damage, lipid peroxidation, and inflammation. It also led to poorer muscle function up to 72 h after exercise in some muscle groups.

Short-term supplementation with vitamin C 2 hours before exercise successfully increased plasma and cellular concentrations, although failed to have any beneficial outcomes in terms of muscle damage or soreness. Supplementation in the hours and days (up to three days) after exercise also produced no beneficial effects, and it may be that supplementation occurred at an inappropriate time. Prolonged supplementation with vitamin C proved more promising (14 days), and was associated with reduced plasma concentrations of interleukin-6 and malondialdehyde. Furthermore, there were modest benefits to certain aspects of muscle soreness and function, although these were not always statistically significant. However, there was no effect on circulating markers of muscle damage (creatine kinase and myoglobin).

These findings suggest that the regular ingestion of vitamin C may be associated with some favourable changes following damaging exercise. However, the consumption of large amounts of vitamin C immediately before or after exercise offer no appreciable benefits, despite large changes in plasma concentrations of this vitamin.

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PUBLICATIONS

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The findings of some of the studies reported in this thesis have been published as follows:

Published papers:

Thompson, D., Nicholas, C., Williams, C. (1999) Muscular soreness following prolonged intermittent high-intensity shuttle running *J. Sports Sci.* **17**: 387-395

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CHAPTER ONE

INTRODUCTION

From a historical perspective, exercise-induced muscle soreness is a well-established field of exercise physiology, and has received the attention of the scientific community for almost a century (Hough 1902). This is likely to be a reflection of the frequency with which muscle soreness occurs, since most people have probably been affected by this condition at one time or another. Indeed, it is difficult to find someone who cannot recollect at least one vivid and painful memory of a particularly serious episode of exercise-induced muscle soreness. The distinguished physiologist A. V. Hill once mused over his memories of muscle soreness, which remain surprisingly pertinent fifty years later:

> '....when I was in good training for long-distance running, if I ran 100 yards several times at top speed I could become unpleasantly stiff. This stiffness could never be cured by anything I did - I could only wait several days till it passed off. I was not incapacitated by such stiffness, but trying to "run it off," as people suggested, did no good - indeed I became all the more stiffer afterwards.... I know of nothing that prevents it except previous training, nor anything that quickens its disappearance; when others have asked me, I have often been forced with shame to confess ignorance.'

Although Hill (1951) made these comments without the hindsight afforded by more recent work, the notion that soreness is the inevitable consequence of unaccustomed physical exertion remains unchanged fifty years later. Importantly, although soreness is a condition that will naturally subside, it has the potential to be debilitating. For the serious athlete, this may prevent optimal training and performance, and therefore has important implications. For the recreational athlete, the sensation of soreness is likely to be more frequent and severe, and represent an unwelcome side effect of exercise.

Muscle soreness is believed to be a symptom of micro-injury to tissues, since sore muscles show considerable disruption to muscle architecture (Friden et al. 1981; Newham et al. 1983b). Muscle damage may be the result of both mechanical and

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metabolic factors, although the principal cause of muscle damage has not been fully resolved (Armstrong 1984, Clarkson and Sayers 1999). Eccentric activity, which is characterised by high peak forces, appears to produce greater soreness and damage than concentric activity (Talag 1973; Newham *et al.* 1983b). However, any unfamiliar exercise will produce a considerable degree of muscle damage, in spite of the absence of an eccentric component (Prou and Marini 1997). One theory that has been proposed, is that muscle damage is the consequence of an exercise-induced elevation in free-radical production (Sjödin *et al.* 1990; Kanter 1994; Jackson 1996). Free-radicals are short-lived chemical species that are able to damage cellular components, and ultimately contribute to cell death (Wu *et al.* 1996). There is the potential for considerable free-radical production during exercise, and free-radicals have been proposed to be an initial cause of exercise-induced muscle damage (Zerba *et al.* 1990b). Furthermore, the extent of initial muscle damage appears to increase after the cessation of exercise (Friden *et al.* 1983; Kuipers *et al.* 1983; Newham *et al.* 1983b), which may also be free-radical related (Duarte *et al.* 1993, Best *et al.* 1999).

There is an elaborate antioxidant defence system in muscle tissues, which enables the effective quenching of free-radicals before they damage cellular components. This system is comprised of a number of antioxidant enzymes and compounds, which are found in various cellular compartments. Several of these compounds cannot be synthesised, but are acquired through the diet (e.g. vitamin E and vitamin C). However, it appears that endogenous systems are overwhelmed during and after certain forms of exercise, since markers of free-radical damage increase immediately (Kanter *et al.* 1993; Child *et al.* 1998c), and several hours after exercise (Maughan *et al.* 1989; McBride *et al.* 1998). The role of free-radicals in the damage process has been confirmed by studies showing that the inhibition of free-radical production subsequently reduces muscle damage (Duarte *et al.* 1993). Additionally, muscle damage may be ameliorated through the provision of an agent with free-radical scavenging properties (Zerba *et al.* 1990b), which also offers considerable benefit to muscle soreness (Krotkiewski *et al.* 1994). One of the more popular supplements has been vitamin E, which is extremely efficient in hydrophobic compartments (Niki 1996). Vitamin E has been demonstrated to reduce certain markers of muscle damage after

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demanding exercise in both animals (Jackson *et al.* 1983) and humans (McBride *et al.* 1998).

Although vitamin E has some distinct benefits, it does not appear to affect ultrastructural damage within cells, only muscle damage associated with membrane permeability (Van Der Meulen *et al.* 1997). Importantly, this may be unrelated to its antioxidant property, since vitamin E also stabilises membranes through a direct interaction with phospholipids (Niki 1996). Additionally, vitamin E must be accumulated in membranes in order to function as a cellular antioxidant, and therefore a long period of supplementation is required. Prolonged supplementation would offer little consolation to the majority of people who engage in unaccustomed exercise, since such activity is typically performed without the benefit of several weeks' foresight. Consequently, it would be more advantageous if an antioxidant could be delivered rapidly, and therefore close to a damaging bout of exercise.

Vitamin C is a water-soluble antioxidant, and in theory, supplementation need not be a long-term affair. Furthermore, vitamin C also has the capacity to indirectly scavenge radicals in hydrophobic compartments, through its ability to recycle vitamin E (Chan 1993). Therefore, vitamin C is probably a major antioxidant, a view which has been supported by *in vitro* findings (Frei *et al.* 1989). However, despite the apparent potential for vitamin C, there is a paucity of research relating vitamin C supplementation to exercise-induced oxidative stress and muscle damage (Sen 1995; Goldfarb 1999). Of the few studies available, vitamin C has been shown to offer some protection against muscle soreness and free-radical damage (Kaminski and Boal 1992; Vasankari *et al.* 1998). Interestingly, plasma levels of vitamin C show an acute increase immediately after exercise (Gleeson *et al.* 1987; Duthie *et al.* 1990). However, there is a fall in the plasma levels of vitamin C in the days after physical stress (Shukla 1969; Hume *et al.* 1972), including after demanding exercise (Gleeson *et al.* 1987). Although speculative, it is tempting to suggest that this reflects increased consumption of this antioxidant due to exercise-induced free-radical production.

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The investigations that are reported in this thesis examined the impact of vitamin C supplementation on exercise-induced muscle soreness and damage. Following a review of the relevant literature (Chapter 2), there is an outline of the experimental procedures that were common between investigations (Chapter 3). There are many potential exercise models for the investigation of muscle soreness and damage (Friden *et al*. 1983; Newham *et al*. 1983b; Fielding *et al*. 1993). However, it was the intention at the outset of this thesis to make the exercise model as representative of 'real life' recreational activities as possible. Some of the most popular activities are examples of multiple-sprint sports (Williams 1990), and there are anecdotal reports of severe soreness following a laboratory-based test designed to simulate such sports (Nicholas 1996). Therefore, the first experimental chapter reported in this thesis determined whether the soreness following this test was of a sufficient intensity for this model to form the basis of subsequent studies (Chapter 4). The following three chapters report a series of investigations where vitamin C was consumed at various times before and after this form of exercise (Chapters 5-7). In the study reported in Chapter 5, vitamin C was consumed two hours before exercise, whereas in the study reported in Chapter 6, vitamin C was consumed over the few days after exercise. The final investigation assessed the effect of a prolonged two-week period of supplementation before exercise (Chapter 7). The thesis is concluded in Chapter 8, with some reflection on the wider implications of the findings reported in each experimental chapter.

CHAPTER TWO

REVIEW OF LITERATURE

2.1 Introduction

Performance of regular exercise produces considerable adaptation, particularly within muscles. The adaptive process allows the progression onto higher and higher levels of work, and is one of the fundamental principles of training. However, the adaptive response is not achieved without muscle working at a higher level than normal - the principle of overload. A moderate increase in the volume of exercise will lead to the beneficial outcome of adaptation. However, if the level of work in a single exercise session is too far above that to which the muscle is accustomed, there will be some degree of damage. Such overexertion probably has little impact in the long-term, but in the short-term it delays the natural adaptive process, in addition to producing the sensation of muscle soreness. In an ideal situation, the gains from a single bout of exercise would be maximal, while factors such as soreness would be minimal.

2.2 Exercise-induced muscle damage

The notion that severe exercise has the potential to damage muscle is not a new one. Almost 100 years ago, Hough (1902) proposed that certain forms of exercise had the capacity to rupture muscle components, and that this was responsible for the soreness that developed several hours later. Hough (1902) also pointed out that muscle soreness which developed several hours after exercise, nowadays frequently referred to as delayed-onset muscle soreness, is very different to the soreness and pain sometimes felt immediately after exercise. Almost fifty years later, A. V. Hill proposed that the injury was likely to be on a microscopic level, and that the process of repair was similar to that for other minor injuries (Hill 1951).

2.2.1 Morphological changes

The clearest evidence that certain forms of exercise have the capacity to damage muscle tissue comes from studies which have directly observed ultrastructural changes. Electron microscopy of biopsies taken after a number of different exercises have revealed considerable changes in muscle architecture (Friden *et al.* 1981; Friden *et al.* 1983; Newham *et al.* 1983b; Hagerman *et al.* 1984; Warhol *et al.* 1985; Friden *et al.* 1988; Fielding *et al.* 1993). The mode of exercise and the criteria used to define damage have varied from study to study, and biopsies have been taken at different time points, although the overall findings appear to confirm structural changes at the level of the muscle fibre. Several studies have reported damage or 'streaming' of the Z band (Friden *et al.* 1981; Friden *et al.* 1983; Fielding *et al.* 1993) and in certain circumstances complete disruption to sarcomeres (Friden *et al.* 1983; Newham *et al.* 1983b; Warhol *et al.* 1985). Other observations include damaged or swollen mitochondria (Friden *et al.* 1983; Hagerman *et al.* 1984; Warhol *et al.* 1985), the presence of empty myotubes (Warhol *et al.* 1985), infiltrating leucocytes (Hagerman *et al.* 1984; Fielding *et al.* 1993), and completely disrupted fibres (Hagerman *et al.* 1984). Such ultrastructural changes have been seen after marathon running (Hagerman *et al.* 1984; Warhol *et al.* 1985), downhill running (Fielding *et al.* 1993), sprinting (Friden *et al.* 1988), running downstairs (Friden *et al.* 1981) eccentric cycling (Friden *et al.* 1983) and bench stepping (Newham *et al.* 1983b). It is important to appreciate, however, that some degree of ultrastructural abnormality is observed in resting individuals (Hagerman *et al.* 1984; Fielding *et al.* 1993), including those who may have performed no activity for up to one week prior to biopsy (Friden *et al.* 1988).

Although muscle biopsies are extremely useful in identifying whether muscle damage has taken place, this technique cannot be applied throughout whole muscle. Kuipers *et al.* (1983) found that after moderate intensity exercise in rats, damage was restricted to small sections of muscle fibres (150-1250 μm), and did not appear to affect whole fibres. Therefore, there is the danger that a biopsy might over- or under-estimate the magnitude of damage, depending on location. Furthermore, biopsies are restricted to certain muscle groups, and only a limited number of samples may be taken.

Nevertheless, biopsies have provided the most compelling and direct evidence that muscle damage does indeed occur following certain forms of exercise.

2.2.2 Circulating levels of muscle proteins

It has become increasingly common to use the appearance of muscle proteins in the circulation as an indirect indication that muscle has been damaged. It has been clear for many years that strenuous exercise leads to increased levels of circulating muscle proteins (Gardner *et al.* 1964; Vejjajiva and Teasdale 1965; Fowler *et al.* 1968; Buyze *et al.* 1976). Muscle enzymes such as creatine kinase (CK), lactate dehydrogenase (LDH), and aspartate aminotransferase (AST) have been commonly used (Roti *et al.* 1981; Newham and Jones 1983; Maughan *et al.* 1989), although it is also possible to detect other proteins such as myoglobin, troponin, and myosin heavy chain fragments (MHC) (Mair *et al.* 1992; Sorichter *et al.* 1997). Proteins such as myoglobin and troponin are ubiquitous in muscle tissue, and so too are enzymes such as CK (Table 2.1).

	Liver	Cardiac Muscle	Skeletal Muscle
CK	0.7	350	2030
LDH	145	124	147
AST	59	52	36

Table 2.1   Enzyme activities in liver, cardiac muscle and skeletal muscle ($U. g^{-1}$) Taken from Buyze *et al.* (1976)

Theoretically, the release of large molecular weight proteins indicates damage to muscle fibres sufficiently great enough to allow their escape through the muscle fibre membrane. In order to appreciate the size of these molecules, the molecular weight (daltons) of some of the commonly used muscle proteins are given below (Mair *et al.* 1992; Sorichter *et al.* 1997):

- Myoglobin 17 800
- Troponin I 18 500
- CK 81 000
- AST 93 000
- LDH 120 000
- MHC 230 000

It has been suggested that proteins are released at different rates according to their molecular weight (Evans and Cannon 1991). However, although Jackson *et al*. (1991) found a variable rate of release of different proteins from incubated rat muscle preparations after damage, this was independent of the size of the molecule. These authors attributed the variation in protein efflux to different levels of intracellular binding. After release into the interstitium, large proteins are transported through the lymph into the circulation (Lindena *et al*. 1979). This process may take some time, as Volfinger *et al*. (1994) found that after an intramuscular injection of CK in horses, there was a delay of several hours before CK appeared in the blood. Once in the blood, different proteins are removed at different rates. Creatine kinase has a half-life of more than 15 h in the circulation (Neumeier *et al*. 1981), whereas myoglobin has a half-life of less than 9 min (Klocke *et al*. 1982).

There are a number of potential problems that arise when using circulating cytosolic muscle proteins as an indication that muscle tissue has been damaged. Entry into the circulation relies principally upon disturbance, in one way or another, to the outer muscle fibre membrane. Therefore, if the sarcolemma remains intact, it is possible that there may be damage within the cell without protein release. Conversely, if the sarcolemma is just momentarily ruptured, there may theoretically be considerable release of proteins without more extensive damage being present. Studies of humans have estimated the mass of damaged muscle to be as high as 5 kg after a marathon by using circulating levels of muscle enzymes (Apple and Rhodes 1988). This rather unrealistic estimate is probably partly due to inaccuracies in the calculation of the rate of CK clearance (Volfinger *et al*. 1994). Even so, studies of rats have suggested that the amount of damage calculated through the use of circulating CK overestimates the true

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magnitude of damage (Kuipers *et al.* 1985; Van Der Meulen *et al.* 1991; Komulainen *et al.* 1995). However, these studies came to this conclusion by measuring ultrastructural damage in a few selected muscles, and relating it to circulating CK levels. It may not be too surprising that measurement of a whole-body parameter such as CK, does not relate well to damage in individual muscles, as other muscles may have been a source of some of the CK. Nevertheless, it is likely that using circulating muscle enzymes in an attempt to quantify the extent of damage does lead to slight overestimation, since it is unlikely that the release of cytosolic proteins is indicative of complete loss of cell viability (Jackson *et al.* 1991). In spite of this, a loss of proteins which are essential to muscle structure and function is likely to reflect some degree of damage, as muscle cells could not continue to lose proteins indefinitely. This is supported by the fact that people who are accustomed to a particular activity show no appreciable rise in blood-borne muscle proteins after exercise (Byrnes *et al.* 1985; Clarkson and Tremblay 1988; Brown *et al.* 1997b).

In the past, questions have been raised about the specificity of circulating levels of proteins, which were assumed to be released from skeletal muscle. As Table 2.1 suggests, enzymes such as CK and AST are found in large amounts in tissues such as the liver and the heart, in addition to skeletal muscle. This concern was supported by the observation that cardiac CK isoenzymes (CK-MB) were elevated following demanding exercise (Robinson *et al.* 1982; Noakes *et al.* 1983). Recent evidence, however, demonstrates that more than 95% of circulating CK is derived from skeletal muscle after demanding exercise (Noakes *et al.* 1983; Hortobágyi and Denahan 1989; Nosaka and Clarkson 1996).

A further potential problem which may arise when dealing with blood-borne muscle proteins, is that of large variability. Extreme variation in circulating levels of muscle proteins has been reported on numerous occasions following damaging exercise (Newham and Jones 1983; Mair *et al.* 1992; Nosaka and Clarkson 1996). Some authors have attempted to classify individuals as low and high responders (Maughan *et al.* 1989), although it generally appears that there are a wide range of possible responses to the same exercise bout. Low values are not the result of some unknown factor in the

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blood inhibiting the activity of creatine kinase (Clarkson and Ebbeling 1988), and may simply be related to different levels of familiarity with a particular form of exercise (Byrnes *et al*. 1985). Additionally, quite large variation in circulating levels of muscle proteins may reflect only small differences in the true extent of muscle damage (Volfinger *et al*. 1994).

Support for the continued use of cytosolic proteins as markers of muscle damage has been provided by recent studies showing myosin heavy chain fragments and skeletal troponin in the circulation after damaging exercise (Mair *et al*. 1992; Prou and Marini 1997; Sorichter *et al*. 1997; Koller *et al*. 1998). Structurally bound proteins are not open to the same criticism as cytosolic proteins, and damage must be more widespread than simply the sarcolemma. Myoglobin is 97% soluble in skeletal muscle cytosol, and CK 87%; whereas there are only very small pools of soluble troponin (3.4%) and myosin heavy chain fragments (0.1%) (Sorichter *et al*. 1997). It has been reported that the increase in CK mirrors that of troponin (Sorichter *et al*. 1997), and a good relationship has been found between circulating myosin heavy chain fragments and CK (Mair *et al*. 1995; Prou and Marini 1997). Therefore, although there are questions over the use of circulating levels of muscle proteins as a means to measure the volume of damaged muscle, they appear to be extremely useful as a reflection of some level of muscle cell damage.

2.2.3 Muscular soreness

Early investigations concentrated on the cause of muscle soreness, and led to the suggestion that muscles were injured in some way (Hough 1902). Although there is a lack of direct evidence that damage is the cause of soreness, there is considerable evidence showing that muscles which are sore are also damaged (Friden *et al*. 1981; Newham *et al*. 1983b; Hagerman *et al*. 1984). Therefore, soreness may not only be an unwelcome side effect of muscle damage, but may also be a useful measure of the extent of muscle damage. Indeed, a recent review of the methods used in the past to investigate muscle damage found that soreness has been the most commonly used marker of injury to date (Warren *et al*. 1999).

There are a number of neurones responsible for conveying the sensation of pain to the brain (Stimmel 1983). Type I and II myelinated afferent fibres, which originate in muscle spindles and golgi tendon organs, do not respond to noxious stimuli, and are therefore unlikely to be involved in muscle soreness (O'Connor and Cook 1999). Type III and type IV afferent fibres are abundant in the connective tissue between fibres and in regions close to capillaries, which are excellent positions for receiving algesic substances released from damaged skeletal muscle (Armstrong 1984; O'Connor and Cook 1999). These originate in muscle as free-nerve endings, or nociceptors, and respond to chemical, mechanical and thermal stimuli (Stimmel 1983). Although many nociceptors are specific for one type of stimulus, many are polymodal, and respond to several types of stimuli (Stimmel 1983). Type III and type IV nociceptors carry dull, aching pain, making these fibres the primary candidates responsible for the sensation of soreness (Armstrong 1984; Ebbeling and Clarkson 1989). Type III fibres respond preferentially to changes in pressure, whereas type IV fibres respond to noxious chemicals (O'Connor and Cook 1999). An interesting quality of nociceptors is that rather than becoming de-sensitised after prolonged stimulation, they actually become more sensitive, lowering their activation threshold (Stimmel 1983).

Damaged muscle is likely to contain a number of agents with the capacity for creating pain. There are a wide range of chemical mediators, including kinins (Dray and Perkins 1993), prostaglandins (Norman and Litwack 1987; Smith 1991), potassium and/or sodium (Graven-Nielsen *et al.* 1997), serotonin (Armstrong 1984), and histamine (Armstrong 1984). Some of these mediators activate nociceptors directly, whereas others sensitise nociceptors, and decrease their activation threshold to some other agent. Furthermore, there is considerable interaction between these mediators, and production of one may lead to increased production of another. For example, bradykinin formed by tissue and plasma kallikreins (in response to tissue trauma, inflammation, hypoxia or low pH), is not only capable of directly sensitising nociceptors, but also induces prostaglandin and cytokine production in macrophages (Dray and Perkins 1993). It is partly for this reason that bradykinin has been suggested to be the most potent algesic (O'Connor and Cook 1999). Increased local prostaglandin concentration may also be one of the major stimuli (Smith 1991). However, the release of prostaglandin E_2 occurs

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immediately after damage to mouse muscle (McArdle *et al.* 1994), at a time when hyperalgesia is absent. This may demonstrate the need for several stimuli, or the prolonged presence of such stimuli, before the sensation of soreness develops.

Mechanical and thermal factors may also have a role in the aetiology of soreness, particularly in conjunction with some of the chemical mediators outlined above. Mechanical stimulation of nociceptors is likely to be in the form of distortion to muscle tissue due to oedema (Armstrong 1984), as a result of either an increased osmotic drive from myofibre debris, or a localised inflammatory response. Friden *et al.* (1986) offered some support for this hypothesis, as damaged muscle showed a considerable increase in intramuscular pressure at a time when soreness was also elevated. However, Graven-Nielsen *et al.* (1997), using an *in vivo* model, reported that increased intramuscular pressure did not increase pain. Although many of these stimuli are able to sensitise nociopceptors directly, they may play an equally important role in facilitating sensitisation by some other agent. It may be this factor that explains why soreness is felt more intensely upon movement or palpation, whereby chemical factors sensitise nociceptors to mechanical distortion and increased intramuscular pressure.

A major difficulty in the assessment of soreness is that it is necessary to gauge what another person is feeling. The most popular method for doing this, is to simply ask subjects to rate the intensity of soreness on some sort of linear scale. Studies in the past have used scales ranging from 0-3 (Abraham 1977), to more complex scales comprising 12 levels of soreness with an extra -10 to 10 dimension (MacIntyre *et al.* 1996). Scales are frequently complemented by adjectives describing the intensity of soreness (Maughan *et al.* 1989), although some investigators prefer not to give any guidance and use a simple 10 cm unmarked line (Bobbert *et al.* 1986). Subjects are often asked to palpate muscles to stimulate the sensation of soreness (Kuipers *et al.* 1985; Kaminski and Boal 1992), or to assess soreness whilst a pre-determined amount of pressure is applied at particular sites (Tiidus and Ianuzzo 1983). It is also possible to determine the level of applied force that is required to elicit the sensation of pain (Edwards *et al.* 1981a; Newham *et al.* 1983a). Tiidus and Ianuzzo (1983) also asked subjects to rate

soreness whilst performing a contraction of a particular muscle group at a pre-determined intensity.

Soreness undoubtedly arises as a consequence of previous exercise, and therefore shares the same origin as exercise-induced muscle damage. An important consideration, however, is that the perception of pain is not always directly related to the amount of tissue damage (O'Connor and Cook 1999). Furthermore, factors other than damage also play a role, such as personality, expectations, and previous experiences (MacIntyre *et al*. 1995; O'Connor and Cook 1999). Nevertheless, soreness can be assessed in both a reliable and valid manner (O'Connor and Cook 1999), and is probably a useful tool. Soreness is sometimes questioned because of its subjectivity, and its failure to relate to more objective measurements, such as changes in muscle structure determined by magnetic resonance imaging or circulating muscle proteins. However, O'Connor and Cook (1999) highlight that if pain is to be investigated, then there is no alternative to subjectivity, since pain is subjective.

2.2.4 Muscle function

Early experiments established that sore muscles show decreased ability to generate maximal voluntary strength (Talag 1973). Initially, it was suggested that the decrease in the ability to generate the same level of force was due to inhibition caused by the pain experienced in damaged muscles (Talag 1973). However, there are numerous reports that sore and damaged muscles have disturbed function at low frequencies of percutaneous electrical stimulation (Edwards *et al*. 1981b; Newham and Jones 1983; Newham *et al*. 1983a; Sargeant and Dolan 1987; Child *et al*. 1998a), indicating damage to the excitation-contraction coupling mechanism. Furthermore, Saxton and Donnelly (1996) found that superimposing electrical stimulation during a maximal voluntary isometric contraction induced no further gains in force. Therefore, subjects were able to overcome any inhibition due to pain, and the loss of force was a consequence of muscle damage. Not all studies have supported this notion, however, and soreness may be involved in the generation of maximal force. Child *et al*. (1998a) recently reported that electrical stimulation of damaged muscles increased the level of force produced,

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whereas before exercise this was not the case. This suggests that, at least in these subjects, the presence of pain limited the amount of force produced. A possible explanation for the discrepancy in these results may be different levels of subject motivation. In spite of this potential drawback, it has been suggested that muscle force may be the best indicator of muscle damage in both humans (Warren *et al.* 1999) and animals (Faulkner and Brooks 1997).

Early studies were restricted to the assessment of muscle function using maximal muscle strength (Talag 1973). The increased availability of isokinetic equipment has provided greater opportunity for the assessment of muscle damage using this potentially useful tool. Maximal voluntary isokinetic testing has excellent reliability (Kannus 1994; Pincivero *et al.* 1997), and has been used recently in the assessment of muscle damage (Friden *et al.* 1983; Mair *et al.* 1992; Prou and Marini 1997; Child *et al.* 1998a). There have been reports that torque after damaging exercise is decreased for longer when assessed at faster isokinetic speeds (Friden *et al.* 1983), and these authors suggested that this technique may be sensitive enough to identify damage in different fibre types.

2.2.5 Other indices of muscle damage

There are many other techniques available for the detection of muscle injury after damaging exercise, but a detailed description is beyond the scope of this review. Abnormalities have been found in damaged muscle using magnetic resonance imaging (Mair *et al.* 1992; Sorichter *et al.* 1995; Nosaka and Clarkson 1996) and magnetic resonance spectroscopy (Zehnder *et al.* 1999). Kao *et al.* (1998) also detected damaged muscles accidentally, using the tracer technetium-99m and single proton emission tomography during a routine bone scan. This approach has been used in the past after damaging exercise, in conjunction with some of the other techniques described above (Newham *et al.* 1986; Crenshaw *et al.* 1993). It has been suggested that the uptake of technetium-99m by muscle is a reflection of damage to the sarcolemma (Newham *et al.* 1986).

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2.3 Consequences of muscle damage

Although soreness generally lasts only a few days, the decline in force after damaging exercise may persist for up to 3 weeks (Pearce *et al.* 1998). Such pronounced effects clearly have the potential to prevent optimal performance. Maximal power output during a 20s all-out test on an isokinetic cycle ergometer appears to be impaired for several days after damaging exercise (Sargeant and Dolan 1987). Additionally, performance of an incremental cycling test (Gleeson et al. 1998a), or steady-state cycling (Gleeson et al. 1995b), is associated with higher heart rate, perceived exertion, respiratory exchange ratio, and lactate concentrations 48h after damaging exercise. Damaging exercise may also affect the availability of appropriate metabolic fuels for prolonged activity. Eccentric exercise of one leg, followed by cycling to deplete glycogen, leads to lower levels of glycogen storage for several days in the leg that exercised eccentrically (Costill *et al.* 1990). Early observations suggested that eccentric exercise decreased muscle GLUT4 transporter content (Asp *et al.* 1995), although recent observations after a marathon suggested that lower levels of glycogen resynthesis could not be explained by this phenomenon (Asp *et al.* 1997). An alternative explanation may be competition between infiltrating leucocytes and muscle cells for glucose (Costill *et al.* 1990). Experiments *in vitro* have supported this hypothesis, and have shown dramatic increases in glucose uptake in mouse muscle incubated with inflammatory cells (Shearer *et al.* 1988).

Therefore, damaging exercise may affect performance of both short-term maximal activities, and prolonged exercise. Furthermore, skilled performance may also be impaired after damaging exercise. Saxton *et al.* (1995) reported increased muscle tremor and poor proprioception after a damaging bout of exercise, which is consistent with the idea that muscle spindles and golgi tendon organs are damaged. Furthermore, Pearce *et al.* (1998) found that performance of a visuomotor tracking task, one method of assessing motor skill, was poorer up to 14 days after damaging exercise.

2.4 Mechanisms responsible for exercise-induced muscle damage

Throughout the one hundred years since Hough (1902) first began to investigate the subject of exercise-induced muscle soreness, a number of mechanisms have been proposed to be the major cause. Exercise intensity and duration both undoubtedly play a role, with intensity possibly being the most important factor (Tiidus and Ianuzzo 1983). It is more typical, however, to consider the damage process as being initiated by either mechanical or metabolic factors (Armstrong 1984; Ebbeling and Clarkson 1989).

2.4.1 Evidence in support of mechanical factors

A number of studies have found that damage and soreness is greater after eccentric, rather than concentric muscle actions (Talag 1973; Newham and Jones 1983; Schwane *et al.* 1983; Sorichter *et al.* 1997). This is probably the strongest evidence supporting mechanical factors as being responsible for exercise-induced muscle damage. At similar workloads, the energy cost of eccentric actions is lighter, but the force generated per fibre greater, than during concentric actions (Newham *et al.* 1983a).

Ultrastructural observations demonstrate that there is considerable disruption to muscle architecture following certain forms of exercise (Section 2.2.1). Damage of this nature after eccentric exercise may be the product of either the high peak forces or long muscle lengths generated during these movements. Lieber and Friden (1993) addressed this question using a rabbit muscle model, and suggested that peak forces accounted for only 8% of the loss in force, whereas the relative increase in muscle length explained 50% of the loss in force. This has lead to the theory of sarcomere 'popping', whereby actin and myosin filaments are pulled so far apart that the Z band is damaged and other passive structures provide support. There is evidence to suggest that this may occur in humans, as eccentric exercise at long muscle lengths causes greater damage and soreness than eccentric exercise at short muscle lengths (Child *et al.* 1998b). Eccentric exercise at long muscle lengths also places considerable strain on the cytoskeleton (Waterman-Storer *et al.* 1991; Lieber *et al.* 1996). Disruption to the cytoskeleton could also disrupt sarcomeres (Waterman-Storer *et al.* 1991), and damage to the cytoskeletal

protein desmin has been observed in the first few minutes of eccentric exercise (Lieber *et al*. 1996). Mechanical stresses may also rupture the sarcolemma, causing considerable damage to the area close to such damage (see Section 2.4.3). However, indirect markers of sarcolemmal integrity (e.g. creatine kinase) do not peak until several days after the most damaging forms of repetitive eccentric activity (Newham and Jones 1983; Jones *et al*. 1986). The explanation for this is not clear, but it may suggest that the initial cause of muscle damage in repetitive eccentric activity is not linked to disruption of the sarcolemma.

Evidence is also accumulating that connective tissue is damaged after certain activities. Damage to this tissue is a particularly attractive explanation for soreness, since type IV nociceptors are abundant in connective tissue (Armstrong 1984). An increased level of urinary hydroxyproline, a marker of collagen turnover, has been reported following damaging exercise (Abraham 1977; Brown *et al*. 1997a). However, there are difficulties in using this method, since only 10% of the hydroxyproline released is excreted, and increased levels may reflect either increased degradation or synthesis (Abraham 1977). Nevertheless, pain after eccentric exercise appears to be greater in the musculo-tendinous junction, and it is possible that this may be a site of considerable damage (Newham *et al*. 1983a).

Eccentric exercise also elevates muscle temperature by more than 1 °C in relation to equivalent concentric activity at a similar workload (Sargeant and Dolan 1987). It is not clear whether this is due to increased heat production or decreased heat removal. Nonetheless, according to Armstrong *et al*. (1991), an increase of this magnitude (due to the Q_{10} effect) could increase the rate of lipid and protein degradation by about 18%. This is supported, to some extent, by a recent study using electrically stimulated mouse muscle *in vitro* (Zerba *et al*. 1990a). In this model, increasing muscle temperature by 10 °C increased the extent of injury by about 50%. It is noteworthy, however, that this study increased muscle temperature from 25 °C to 35 °C, which has little physiological relevance.

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2.4.2 Evidence in support of metabolic factors

It appears that exercise is able produce muscle damage when the metabolic demand is low, if the mechanical stress is high (e.g. eccentric actions). If metabolic factors were equally as potent in terms of creating damage, then the reverse would also be true. Demanding cycling has a minimal eccentric component, and can be sustained for long periods of time at a high metabolic rate. There is little increase in markers of muscle damage following races lasting over 5 hours in professional cyclists (Mena et al. 1996). However, this finding is compounded by the use of highly trained subjects, as individuals accustomed to exercise (including eccentric exercise) show little increase in soreness or markers of muscle damage (Byrnes et al. 1985; Croiser et al. 1999). It is highly likely, therefore, that it is partly the unusual and atypical nature of eccentric actions which makes them so damaging. Therefore, as might be expected, prolonged cycling leads to a pronounced increase in circulating markers of muscle damage in non-cyclists (Roxin et al. 1986). Although the increases were much smaller than after some other forms of exercise, it demonstrates that concentric exercise is able to damage muscle tissue, although the site of damage is unknown. This is supported by the finding that normally non-damaging concentric and isometric exercise leads to considerable muscle damage after 42 days of bed-rest (Prou and Marini 1997). Therefore, metabolic factors cannot be dismissed as a potential initial cause of exercise-induced muscle damage.

It has been postulated that ultrastructural muscle damage following a marathon was due to the depletion of energy substrates (Warhol et al. 1985). Although the authors had no firm basis on which to form this conclusion, it is feasible that a severe reduction in cellular ATP may lead to muscle damage. During exercise, muscle cells face the dual demands for ATP from both the contractile apparatus and other ATP demanding processes (e.g. sodium-potassium ATPase). Individuals suffering from McArdle's disease and other metabolic disorders suffer significant muscle damage after exercise, which in extreme cases may lead to rhabdomyolysis (McArdle 1974). Furthermore, West-Jordan et al. (1990) induced damage to rat muscle by incubation with dinitrophenol, which creates metabolic stress by uncoupling mitochondrial oxidative phosphorylation. Interestingly, this study showed that there was a 20 minute delay

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between the loss of ATP and PCr determined through nuclear magnetic resonance, and the efflux of CK. These authors suggested that although a loss of energy may induce muscle damage, it is probably through the initiation of some other process. Kuipers *et al.* (1983) investigated the response of rats to exercise of a mild, concentric nature. After 1h of exercise there was considerable evidence of ultrastructural damage, which the authors attributed to local energy deficiency at the membrane. Other animal studies have found that the extent of damage after a 2.5h uphill run is much greater than that predicted from a 1.5 h run (Van Der Meulen *et al.* 1991). These authors attributed the greater damage to glycogen depletion in the longer duration exercise.

One factor which argues against a lack of ATP as a potential mechanism for muscle damage, is that ATP concentrations are rarely depleted in muscle cells, after either short or long duration fatiguing exercise (Greenhaff *et al.* 1994; Tsintzas *et al.* 1996). A possible explanation for this may be that the biopsy technique is not sensitive enough to detect changes in ATP concentrations. Alternatively, it may be that there is a focal loss of energy, whereby a particular part of a cell is 'starved' of ATP, which is not detected in biopsy samples.

Increasing metabolic rate during exercise is also associated with an increase in the production of free-radicals. Since this will form a major part of this review, it will not be touched upon here, and the reader is referred to Sections 2.6 to 2.9.

2.4.3 Secondary processes as a consequence of initial damage

As the preceding discussion highlights, the initial cause of muscle damage may depend to a great extent on the nature of the exercise. However, whatever the initial cause of damage or disruption to muscle, the sequence of events that immediately follows probably has much in common. A great deal of importance has been attached to intracellular levels of calcium (Rodemann *et al.* 1982; Jones *et al.* 1984; Duncan and Jackson 1987). Intracellular calcium concentrations are very tightly regulated, and a disturbance in calcium homeostasis would have several negative consequences. Concentrations of calcium inside resting muscle fibres are only 0.1 μmol.l^{-1}, whereas

extracellular concentrations are 2-3 mmol.l⁻¹ (Armstrong *et al.* 1991). Duncan and Jackson (1987) showed that the removal of extracellular calcium prevented muscle damage from occurring in incubated mouse muscle in a number of conditions. This not only highlights the importance of calcium, but suggests that increased intracellular concentrations arise as a consequence of increased sarcolemmal permeability, although damage to the sarcoplasmic reticulum may also be a potential source of calcium (Rodemann *et al.* 1982).

Calcium has a number of damage-initiating effects, including the activation of phospholipase, which oxidises phospholipids to arachidonic acid (Section 2.5). This may damage membranes directly, or the resulting lysophospholipids and free-fatty acids may disrupt the membrane because of their detergent like properties (Duncan and Jackson 1987; McArdle and Jackson 1997). Inhibitors of phospholipase have been demonstrated to reduce sarcolemmal damage in incubated animal muscle, even in the presence of calcium (Rodemann *et al.* 1982; Duncan and Jackson 1987). It has been speculated that increased activity of phospholipase leads to increased prostaglandin synthesis from its precursor arachidonic acid, which in turn activates lysosomal proteases (Rodemann *et al.* 1982). As may be expected, therefore, damage to the sarcolemma can be prevented by the inhibition of phospholipase (Duncan and Jackson 1987) or lysosomal proteases (Rodemann *et al.* 1982). However, although an inhibitor of phospholipase offered protection to the sarcolemma, it had little effect on the extent of ultrastructural damage inside the same muscle (Duncan and Jackson 1987).

A number of calcium activated proteases (calpain I and II) have been identified in skeletal muscle, and these may be responsible for structural damage (Armstrong *et al.* 1991; McArdle and Jackson 1997). Apparently, calpain has a great affinity for cytoskeletal proteins such as desmin, whereas actin and myosin are not substrates for calpain (Clarkson and Sayers 1999). The localisation of much of the damage around the Z band may be explained by this observation, since this is the region where cytoskeletal proteins predominate. It has been suggested that ultrastructural damage may occur without enzyme release, but that any stress great enough to cause an efflux of muscle enzymes would also cause damage to ultrastructural components, due to an influx of

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calcium (Duncan and Jackson 1987). Jones *et al.* (1984) also suggested that once calcium became available within the cell, it would rapidly be taken up by mitochondria, inhibiting ATP synthesis and thereby creating metabolic stress. This would further add to the disturbance in calcium homeostasis, since the sarcoplasmic reticulum requires ATP to regulate intracellular calcium. However, West-Jordan *et al.* (1990) found no change in intracellular ATP by NMR when rat muscle was incubated with calcium. Nevertheless, there was an efflux of CK, although this occurred without an appreciable loss of ATP. McArdle and Jackson (1997) proposed that no single process is activated when intracellular calcium levels rise above normal, but there is a sequence of catastrophic processes that lead to cell death. However, as Armstrong (1991) points out, myofibre necrosis is quite rare. Indeed, after an extremely damaging race lasting approximately 20h, including a large amount of downhill running, only 1% of 3698 fibres analysed in sore muscles were necrotic (Crenshaw *et al.* 1993). It is more likely that damaged areas are 'walled-off', and a demarcation membrane formed around the damaged area. This probably explains why only small sections of muscle are affected (Kuipers *et al.* 1983), rather than small areas of damage initiating processes which lead to cell death.

2.5 Inflammation

Evidence is accumulating that damaging exercise provokes several aspects of the inflammatory response. Inflammation is characterised by the movement of fluid, plasma proteins, and leucocytes into tissues following injury or infection (MacIntyre *et al.* 1995).

2.5.1 Evidence of inflammation

Damaged muscles have been reported to feel warm and swollen (Newham and Jones 1983), and swelling may persist for more than one week after exercise (Nosaka and Clarkson 1996). This may be indicative of inflammation, although Friden *et al.* (1986) suggested that swelling may be the consequence of oedema caused by increased intramuscular osmotic pressure due to the release of muscle proteins. Nevertheless,

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more direct evidence is available, and muscle biopsies taken after several forms of exercise have shown increased leucocyte numbers in damaged muscle (Jones *et al.* 1986; Costill *et al.* 1990; Fielding *et al.* 1993; Hellsten *et al.* 1997). Other studies, however, have looked for infiltrating leucocytes at similar time points after equally damaging exercise and failed to find them (Warhol *et al.* 1985; Friden *et al.* 1988). There are several possible explanations for such a discrepancy, although the simplest might be the focal nature of damage, and the likelihood of finding these sites using the biopsy technique. MacIntyre *et al.* (1996) recently adopted a different approach, and harvested leucocytes from their subjects, which were labelled and re-introduced after damaging eccentric exercise of one leg. These authors observed an increase in radiolabel in the exercised leg, but no such increase was seen in the control leg. This was interpreted as increased leucocyte infiltration at the site of damage. It must be pointed out, however, that leucocytes must be present inside either the interstitium or muscle fibres for it to be evidence of inflammation. Nevertheless, if considered collectively with evidence from muscle biopsies, it suggests that damaging exercise leads to infiltration of affected muscles.

There appears to be a relatively greater increase in the circulating numbers of leucocytes after damaging exercise, than after similar non-damaging concentric exercise (Smith *et al.* 1989a; Pizza *et al.* 1995). Exercise increases circulating numbers of leucocytes immediately post-exercise, with further increases in the few hours after exercise (Gleeson *et al.* 1995a). This is accompanied by a fall in the number of circulating leucocytes in the days after damaging exercise, which may reflect margination of leucocytes in the first stages of inflammation (Gleeson *et al.* 1995a). This has been supported by studies showing increased accumulation of intra-muscular neutrophils as soon as 45 minutes after downhill running (Fielding *et al.* 1993). Neutrophils probably live 1-2 days after migrating into tissues (Fielding *et al.* 1993), whereas after this time the leucocytes found in damaged muscle are mostly macrophages (Round *et al.* 1987). It may be that there are two types of macrophage, one involved in the removal of debris, and the other involved in muscle regeneration in conjunction with myogenic cells (Tidball 1995).

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2.5.2 Initial inflammatory processes

Tidball (1995) recently discussed the possibility of there being some sort of 'wound hormone' released by muscle cells immediately after injury (possibly fibroblast or platelet growth factor). However, the time course of inflammation suggests that cells other than myofibres are responsible for initiating the inflammatory response. Tissue fragments and debris may provide the stimulus for resident macrophages and fibroblasts to activate other elements of the immune system (Pyne 1994). Cells such as macrophages are capable of producing a wide range of chemoattractants and other inflammatory mediators, including complement, leukotrienes and various cytokines (de Souza *et al.* 1985). There is some evidence that exercise increases circulating levels of activated complement proteins (Cannon *et al.* 1996), and Castell *et al.* (1997) showed a four-fold increase in C5a immediately after a marathon. An increase in complement would activate chemotaxis and also enhance phagocytosis by both neutrophils and macrophages.

A great deal of attention has been directed towards cytokines and their potential role in inflammation following exercise-induced muscle damage. Cytokines have a role in initiating, regulating, and terminating inflammatory processes (Dinarello 1990; Northoff *et al.* 1994), including facilitating the influx of many different leucocytes into damaged tissue (Pedersen *et al.* 1998). The sequence of events following injury to muscle tissue may involve the initial release of tumour necrosis factor (TNF) and interleukin-1 (IL-1) by resident leucocytes close to the site of damage, which in turn up-regulate the production of interleukin-6 (IL-6) (Pedersen *et al.* 1998). Numerous unsuccessful attempts have been made to detect increased circulating levels of TNF after a variety of different exercises (Haahr *et al.* 1991; Smith *et al.* 1992; Ullum *et al.* 1994; Cannon *et al.* 1996; Castell *et al.* 1997; Ostrowski *et al.* 1998a), although Gannon *et al.* (1997) reported an increase in TNF after a 7h race in elite cyclists. Early studies reported large increases in IL-1 after damaging eccentric cycling in untrained men (Evans *et al.* 1986), although assays used at this time probably measured IL-1 and IL-6 at the same time (Northoff *et al.* 1994). More recent reports on IL-1 are conflicting. Several studies have reported no change in systemic levels of IL-1β after prolonged running (Castell *et al.* 1997; Nehlsen-Cannarella *et al.* 1997; Ostrowski *et al.*

1998a), cycling (Smith *et al.* 1992; Ullum *et al.* 1994), or maximal eccentric exercise of the quadriceps (Hellsten *et al.* 1997). Other investigators, however, have found small increases in IL-1β after a marathon (Ostrowski *et al.* 1998b). Furthermore, a recent study used a sensitive radioimmunoassay and found that circulating levels of IL-1β increased with increasing exercise intensity during prolonged cycling (Bury *et al.* 1996). The discrepancies between studies may be partly due to the small changes which take place, the local nature of these changes, and the short half-lives of cytokines such as IL-1 and TNF (Sipe 1990; Pedersen *et al.* 1998). On a local level, cytokines may be more readily detected, and Fielding *et al.* (1993) reported increased muscle levels of IL-1β after downhill running. Additionally, IL-1β concentration was related to the extent of neutrophil infiltration (Fielding *et al.* 1993). Further support for the role of IL-1 comes in the form of increased levels of IL-1 receptor antagonist (IL-1ra) after prolonged running (Nehlsen-Cannarella *et al.* 1997; Ostrowski *et al.* 1998a; Ostrowski *et al.* 1998b). Since downregulation of IL-1 is activated, it suggests that IL-1 has been secreted at some point during exercise. However, it is feasible that IL-1 is not released, and increased IL-1ra simply reflects induction by increased levels of IL-6.

Whereas the results for IL-1 and TNF are somewhat conflicting, there appears to be greater consensus regarding exercise and IL-6. Various types of exercise have been shown to induce elevated circulating levels of IL-6, including prolonged running (Castell *et al.* 1997; Nehlsen-Cannarella *et al.* 1997; Nieman *et al.* 1997; Ostrowski *et al.* 1998a; Ostrowski *et al.* 1998b), cycling (Ullum *et al.* 1994), and maximal eccentric actions of the quadriceps (Hellsten *et al.* 1997). A recent study suggested that IL-6 is produced in response to damaging exercise, as eccentric cycling increased circulating levels of IL-6, whereas concentric cycling did not (Bruunsgaard *et al.* 1997). This hypothesis is supported by other studies using a concentric cycling model, which did not change systemic levels of IL-6, and did not provoke soreness or an efflux of muscle enzymes (Smith *et al.* 1992). However, concentric cycling of a slightly higher intensity but similar duration has been shown to increase circulating levels of IL-6 (Ullum *et al.* 1994). It has been suggested that exercise increases IL-6 release from circulating leucocytes or those in the spleen (Papanicolaou *et al.* 1996), possibly due to some form of hormonal stimulus. Ostrowski *et al.* (1998b), however, demonstrated that messenger

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RNA for IL-6 is found in muscle tissue after a marathon, but not in circulating mononuclear cells. This suggests that leucocytes and possibly other cells (e.g. endothelial cells) in muscle are the source of increased systemic levels of IL-6, not leucocytes elsewhere.

Once released into the circulation, most cytokines are able to induce the synthesis of a number of different hepatic proteins (such as C-reactive protein). A recent study showed IL-6 to be very important in mediating the systemic acute-phase response (Kopf et al. 1994). In this study, mice with a dysfunctional gene for IL-6 were unable to induce production of acute-phase proteins in response to tissue injury. Acute-phase proteins are involved in the successful identification of damaged areas, and work together with complement to opsonise fragments of damaged tissue and to facilitate removal by phagocytic cells (Sipe 1990). Acute-phase proteins may also serve to protect cells from the effects of free-radicals and proteases released by infiltrating leucocytes (Sipe 1990). Increased levels of serum C-reactive protein (CRP) have been seen after exercise, although generally there is a delay between peak cytokine concentrations and peak acute-phase proteins. Smith et al. (1992) reported increased levels of CRP in untrained subjects after cycling for 1 hour at 60% maximal oxygen uptake ($\dot{V}O_2$ max), but not in trained subjects. Prolonged exercise has been demonstrated to produce a considerable increase in CRP the day after exercise (Taylor et al. 1987; Castell et al. 1997). As would be expected, a damaging bout of bench stepping produced a pronounced rise in CRP the day after exercise (Gleeson et al. 1995a). A recent study reported that a damaging eccentric exercise protocol of the leg flexors and extensors failed to increase levels of CRP (Croiser et al. 1999). However, blood samples were not taken until 48h post-exercise, which is after the time CRP appears to peak (Taylor et al. 1987; Gleeson et al. 1995a).

2.5.3 Inflammation and soreness

It appears, therefore, that at least certain aspects of an inflammatory response are initiated after damaging exercise. This type of response, and the time course which it follows, makes it a likely candidate for much of the soreness felt after damaging

exercise (Section 2.2.3). Injury to tissue is a potent stimulus for the activation of tissue and plasma kallikreins, leading to the synthesis of bradykinin (Dray and Perkins 1993). Bradykinin is not only a potent algesic, but also induces prostaglandin synthesis by macrophages (Dray and Perkins 1993). Prostaglandins have been suggested to be one of the key mediators of soreness after damaging exercise (Smith 1991). The interaction between prostaglandins, soreness, and inflammation is further enhanced by the knowledge that IL-1β, TNF, and IL-6 are able to induce synthesis of prostaglandins, and therefore pain (Ferreira et al. 1988; Dinarello 1990; de Vries et al. 1995). Additionally, Stauber et al. (1990) observed mast cell degranulation in biopsies 48 h after maximal eccentric exercise of the elbow flexors. Mast cells produce histamine, a potent algesic, in addition to prostaglandins. It is conceivable that the presence of macrophages, mast cells, and neutrophils in muscle tissue are largely responsible for the sensation of soreness. These processes, however, probably interact with other factors such as mechanical distortion either due to swelling or muscular contraction.

2.5.4 Muscle damage arising from inflammation

Although some degree of inflammation is probably essential for full recovery from exercise-induced muscle damage (Tidball 1995), the inflammatory response may also contribute to the damage process. In general, the extent of muscle damage appears to increase in the post-exercise period. Muscle biopsies show that ultrastructural abnormalities are greater 1-3 days after damaging exercise, than immediately after exercise (Friden et al. 1983; Newham et al. 1983b). This has been supported by observations in rats, and Kuipers et al. (1983) found that the extent of damage peaked 1-2 days after exercise. Furthermore, circulating levels of muscle proteins rarely peak immediately after damaging exercise, but in the few days after exercise (Newham and Jones 1983; Mair et al. 1992; Gleeson et al. 1995a; Sorichter et al. 1997). Additional evidence comes in the form of a biphasic decline in maximal voluntary force (MacIntyre et al. 1996). In this study, strength declined immediately after exercise (partly due to fatigue), recovered to some extent a few hours later, but then showed a secondary decline the next day. It was postulated that the secondary loss of force was due to damage caused by inflammation. Such tissue damage may occur as a result of the

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release of extracellular products by inflammatory cells, during the removal of host cells and debris as a first step in tissue remodelling, or during the failure to terminate acute inflammatory responses (Smith 1994).

Neutrophils are probably responsible for much of the damage that occurs during an inflammatory response, and this topic has received a great deal of attention recently (Weiss 1989; Pyne 1994; Smith 1994). Neutrophils accumulate in muscle within an hour of damaging exercise (Fielding et al. 1993). Once activated, neutrophils release more than 50 destructive agents into the extracellular medium (Weiss 1989), most of which are not specific for damaged tissue, but also attack healthy tissue. Barbior (1984) applied the following vivid description; 'Unlike cytotoxic lymphocytes and the complement system, which destroy their targets with a drop of poison, the professional phagocytes kill like Attila the Hun, deploying a battery of weapons that lay waste to both the targets and the nearby landscape with the subtlety of an artillery barrage'. Activated neutrophils increase their oxygen consumption by 50-100 fold (the oxidative or respiratory burst), and free-radicals are produced by the single electron reduction of oxygen to form the superoxide radical (Section 2.6.4). Neutrophils also undergo degranulation upon activation, and release many different enzymes (e.g. elastase and myeloperoxidase), which are central to neutrophil function (Pyne 1994). Such enzymes augment the effects of superoxide, and participate in the digestion of micro-organisms and damaged host cells (including muscle). Elevated circulating levels of elastase have been observed immediately after cycling (Smith et al. 1996), and several days after damaging eccentric exercise (Gleeson et al. 1998b). Increased concentrations of myeloperoxidase have been observed after muscle damage induced by ischaemia followed by reperfusion in a rabbit model (Bushell et al. 1996), and after cycling in humans (Pincemail et al. 1990).

2.5.5 Anti-inflammatory agents

Bushell et al. (1996) found that injection of a corticosteroid 30 min before ischaemia-reperfusion injury considerably reduced the extent of ultrastructural damage in rabbits, although the efflux of CK was unaltered. This may have been due to the suppression of

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inflammatory processes, as Papanicolaou *et al.* (1996) found that administration of different corticosteroids several hours before demanding exercise in humans decreased levels of IL-6. This is consistent with the idea that corticosteroids are able to suppress many aspects of inflammation (Goldstein *et al.* 1992). Other studies have attempted to influence the extent of muscle damage and soreness using non-steroidal anti-inflammatory drugs, which inhibit prostaglandin synthesis. Kuipers *et al.* (1985) administered flurbiprofen before and after eccentric cycling, but found no effect on either soreness or markers of muscle damage. However, there were problems with the cross-over design used in this particular study, which led to a repeated-bout effect which was greater than any possible treatment effect. There are similar concerns over a recent study which investigated the effect of naproxen administration before and after weight-lifting exercise (Bourgeois *et al.* 1999). Again there was no effect on either soreness or markers of muscle damage, although it was not reported to what extent repeated exposure to the same exercise might have confounded the results. There are other reports that suggest that naproxen given after damaging exercise may offer some benefit to soreness (MacIntyre *et al.* 1995), although the full results of this study were not reported. However, the administration of ibuprofen, a drug known to suppress prostaglandin, kinin, and histamine synthesis, has been associated with a number of beneficial outcomes (Hasson *et al.* 1993). This study adopted a matched-group design, and ibuprofen was given either before (prophylactic treatment) or one day after (therapeutic treatment) a damaging bout of exercise. Prophylactic consumption of ibuprofen reduced soreness the day after exercise, and two days after exercise soreness was lower in both prophylactic and therapeutic groups. There were similar benefits to the maintenance of muscle force, although there was no effect on circulating levels of CK.
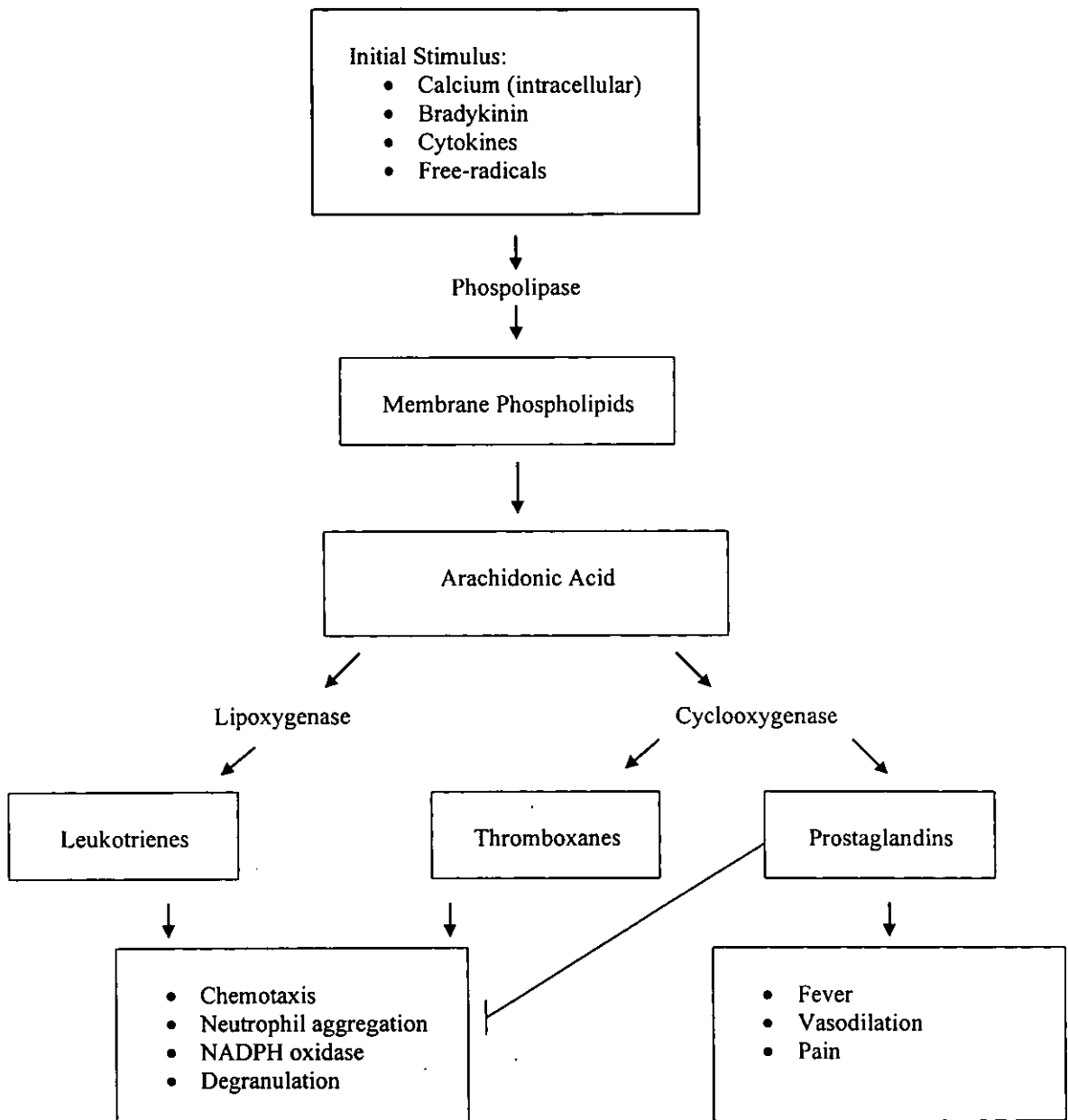
Figure 2.1   Eicosanoid metabolism.
                    Adapted from Fantone (1985)

The majority of studies that have attempted to reduce the extent of inflammation following exercise-induced muscle damage have given cyclo-oxygenase inhibitors, such as ibuprofen and flubriprofen. As Figure 2.1 shows, this affects only one branch of eicosanoid metabolism, and neglects the potent pro-inflammatory lipoxygenase arm of this pathway. It is well established that cyclo-oxygenase inhibitors reduce prostaglandin synthesis, but it is possible that this may divert arachidonic acid to lipoxygenase and

promote leukotriene synthesis (Higgs *et al.* 1985). Glucocorticoids, however, prevent

phospholipase and other inflammatory responses, and therefore have much greater and

wide-reaching anti-inflammatory effects. It may be that anti-inflammatory agents need

to have greater impact on different aspects of acute inflammation, including

lipoxygenase metabolism of arachidonic acid.

2.6 Free-radicals

In general, most chemicals contain paired electrons in their electron orbitals. A free-

radical, however, is any atom or molecule with a lone unpaired electron occupying an

outer orbital (Demopoulos 1973a). The presence of an unpaired electron in the outer

orbital of a free-radical gives it unusual chemical and physical properties. The lone

electron endeavours to interact with other electrons and form an electron pair, and

therefore a chemical bond. It is this property which makes free-radicals extremely

reactive and short-lived, although different radicals may have very different levels of

reactivity (Del Maestro 1980). There are many potential species of radical, depending

on the major atom. A great deal of attention has been focused on oxygen-centred

radicals, and has provided considerable evidence for their role in the initiation of a

number of conditions, including exercise-induced muscle damage.

2.6.1 Oxygen-centred radicals

Fridovich (1978) pointed out that, although aerobic metabolism has great advantages, it

is 'fraught with danger'. Molecular oxygen, which is a relatively stable di-radical, is

capable of initiating and contributing to free-radical reactions (Demopoulos 1973a). As

Figure 2.2 demonstrates, the univalent reduction of oxygen is able to produce

superoxide ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\cdot}$).

$$O_2 \xrightarrow{e^-} O_2^{-\cdot} \xrightarrow{e^- + 2H^+} H_2O_2 \xrightarrow{e^- + H^+} OH^{\cdot} \xrightarrow{e^- + H^+} H_2O$$
$$H_2O$$
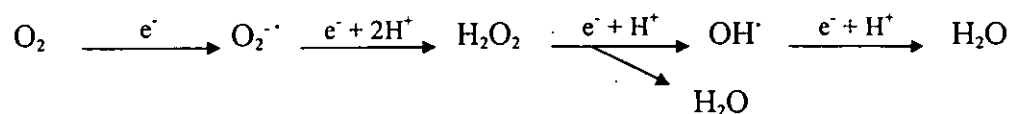
Figure 2.2   Univalent reduction of oxygen.
The dot signifies a radical. Taken from Fridovich (1978).

Hydrogen peroxide is not a true radical, and the term reactive-oxygen species (ROS) may be more appropriate. Although H_2O_2 does not possess an unpaired electron, it is able to participate in many radical processes. Singlet oxygen is a another example of a ROS, and is formed through the excitation of molecular oxygen from a neighbouring chemical reaction. This does not require the addition of an electron to oxygen, but an existing electron 'jumps' into an unpaired state in an outer orbital. Many species of reactive-oxygen rapidly interchange (Equations 1-4), and therefore it is difficult to attribute effects to any one radical. Equation 4 demonstrates the Fenton reaction, and copper (Cu^+) may replace iron for the same net result. Ferrous iron (Fe^{2+}) may be recycled from ferric iron (Fe^{3+}) by molecules such as superoxide, ascorbic acid, and thiols. Other reactive-oxygen species include hydroperoxide (ROOH), the peroxy radical (ROO˙), and the alkoxy radical (RO˙), where R represents any organic molecule. Some ROS are able to diffuse considerable distances (e.g. H_2O_2 and ROOH), whereas others are so reactive (e.g. OH˙) that they are unlikely to travel greater than 1-5 molecular diameters from their site of formation (Pryor 1986). As the preceding discussion demonstrates, once a radical is formed the consequences are unpredictable, since it may travel varying distances depending on how reactive it is, and what other molecules it meets first.

$$O_2^{-˙} + H_2O \rightarrow 2OH˙ \tag{1}$$
$$O_2^{-˙} + 2H^+ \rightarrow H_2O_2 \tag{2}$$
$$O_2^{-˙} + H_2O_2 \rightarrow OH˙ + OH^- + O_2 \tag{3}$$
$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH˙ + OH^- \tag{4}$$

2.6.2 Mitochondria

Complete reduction of a molecule of oxygen via the electron transport chain requires four electrons to produce two molecules of water. Cytochrome oxidase, the final protein of the electron transfer chain, normally reduces oxygen to water in a single tetravalent step and avoids the production of oxygen-centred radicals. However, reduction of oxygen may also occur in a univalent manner, and form oxygen-centred radicals in the process (Fridovich 1978). There are several potential sites where electrons may 'leak-

off' the respiratory chain, with ubiquinone and cytochrome b being likely candidates (Nohl *et al.* 1986). Although the exact site on the respiratory chain has not been clearly identified, it has been known for many years that isolated mitochondria are able to generate reactive-oxygen species (Boveris and Chance 1973). It has been estimated that, in normal conditions, 1-15% of oxygen consumption may be reduced by single electrons derived from mitochondria (Sawyer 1988; Das and Engelman 1990).

Muscle mitochondrial oxygen uptake may increase more than one hundred-fold during demanding exercise (Sen 1995), and therefore free-radical production may increase accordingly. Additionally, alterations in the rate of mitochondrial respiration, which may occur during intermittent exercise, may predispose mitochondria to electron leakage (Del Maestro 1980). Furthermore, mitochondrial radical production may be increased by fatiguing exercise (Davies *et al.* 1982; Shi *et al.* 1999), and an exercise-induced elevation in muscle temperature (Salo *et al.* 1991). Therefore, mitochondria probably represent a site of considerable radical production during certain forms of metabolically demanding exercise.

2.6.3 Xanthine oxidase

In certain situations, the requirement for ATP may be such that the adenylate kinase reaction is activated, leading to the formation of ATP and AMP from 2ADP (Graham *et al.* 1995). Accumulation of AMP may lead to the activation of AMP deaminase, producing IMP, which may subsequently be catabolised to hypoxanthine. In normal conditions, hypoxanthine leaves muscle cells and is oxidised to xanthine, and finally to uric acid, by endothelial cell xanthine dehydrogenase (XDH). As Figure 2.3 demonstrates, XDH uses NAD^+ as an electron acceptor, and no free-radical species are formed in this process. However, it has been suggested that the normal XDH form may be irreversibly converted to a xanthine oxidase (XO) form. This process may occur as a consequence of a calcium activated protease (Mc Cord 1985), or by some form of limited oxidation of the enzyme (Hellsten 1996). Once activated, the XO form uses oxygen as the final electron acceptor, and therefore produces superoxide. This has been proposed as the mechanism for increased free-radical production during ischaemia

followed by reperfusion. Firstly, there is a fall in ATP concentration due to the lack of oxygen for mitochondrial respiration, which increases the concentration of hypoxanthine. This is accompanied by the irreversible formation of XO, and superoxide production (Mc Cord 1985).

Pathway A   Pathway B
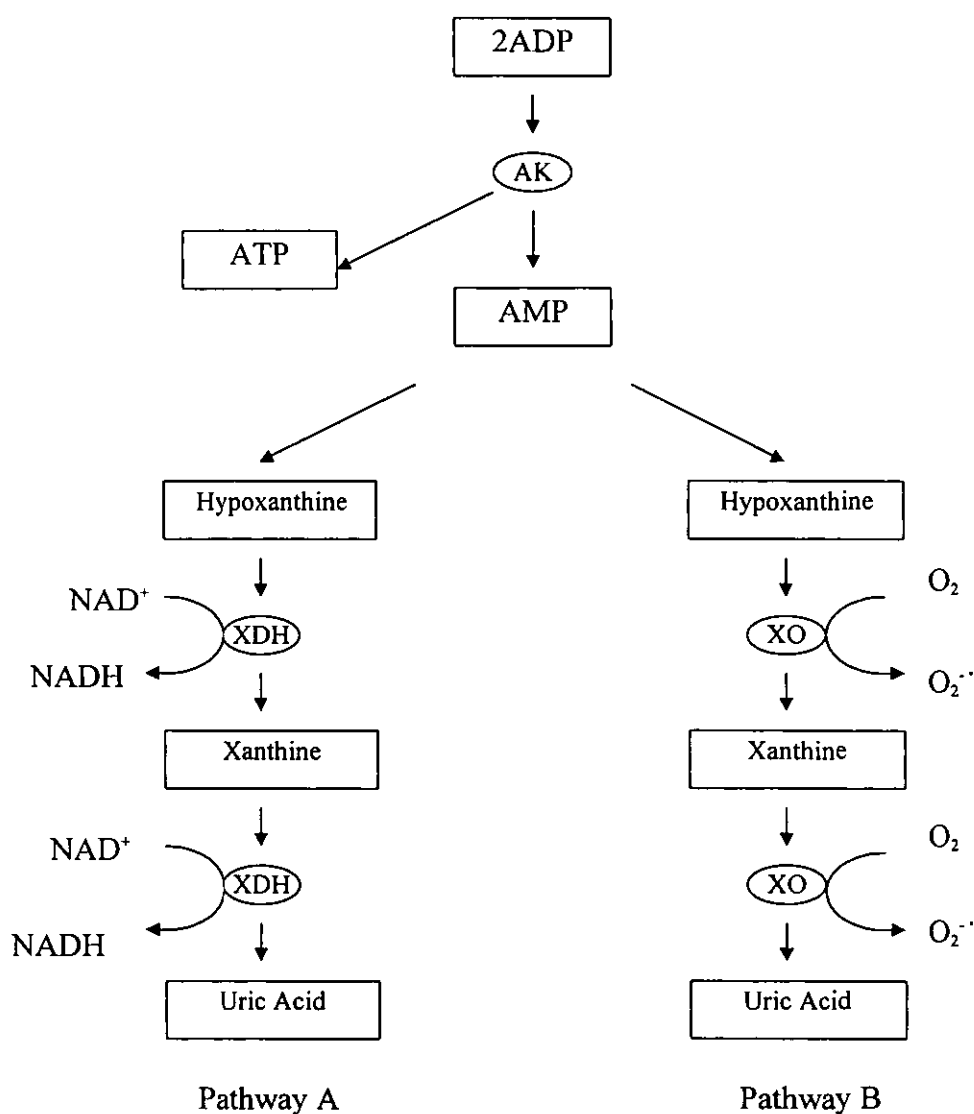
Figure 2.3   The adenylate kinase reaction and subsequent deamination of AMP to IMP (not shown) and oxidation of hypoxanthine and xanthine to uric acid. Normally, hypoxanthine and xanthine are oxidised by xanthine dehydrogenase (XDH), which uses NAD^+ as an electron acceptor (pathway A). However, in certain circumstances (see text), XDH is converted to a xanthine oxidase (XO) form which produces superoxide (O_2^-) by using oxygen as an electron acceptor (pathway B). Adapted from Sjödin et al. (1990).

At exercise intensities above 40% $\dot{V}O_2$ max, AMP deamination increases proportionally (Sahlin and Broberg 1990). There are reports that muscles release hypoxanthine after cycling (Sahlin *et al.* 1991), and after intense exercise of the quadriceps (Bangsbo *et al.* 1992). Therefore, the substrate for xanthine oxidase would theoretically become available, and free-radical processes may become initiated during exercise.

2.6.4 Polymorphonuclear leucocytes

Oxygen-centred free-radicals are also produced by polymorphonuclear leucocytes. Once activated, neutrophils and other phagocytes produce superoxide and hydrolytic enzymes as part of their role in an immune or inflammatory response (Section 2.5). These are produced simultaneously and substantially increase the killing power of phagocytes (Smith 1994). The electrons for superoxide production are provided in the form of NADPH, and a continuous supply of NADPH is fuelled by an increase in the activity of the hexose monophosphate shunt (Barbior 1984). The enzyme responsible for this process has been termed NADPH oxidase, and becomes functional when phagocytes are activated (Barbior *et al.* 1988). Neutrophil oxygen consumption may increase 100 fold (the respiratory burst), and oxygen becomes reduced according to the equation below:

$$2O_2 + NADPH \rightarrow 2O_2^{-\cdot} + NADP^+ + H^+ \tag{5}$$
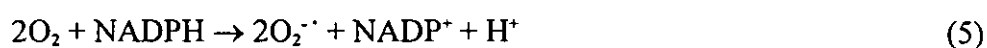
The NADPH oxidase complex is situated across the membrane, so that NADPH binding sites are located facing the cytoplasm, whereas superoxide is released into the extracellular space (Winterbourn 1990). Although superoxide appears to be the only radical generated by NADPH oxidase (Winterbourn 1990), it subsequently leads to the formation of other radicals (Equations 1-4). Furthermore, H_2O_2 may combine with chloride ions to form hypochlorous acid (HOCl), a process catalysed by myeloperoxidase released during degranulation (Weiss 1989). This is a potent and very reactive oxidant, and therefore does not travel far (Winterbourn 1990). It has been suggested that HOCl is the most powerful oxidant produced in large quantities by

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neutrophils (Weiss 1989). As Weiss (1989) points out, the destructive potential of HOCl is fully appreciated when it is considered that HOCl is the active ingredient in household bleach.

Infiltrating leucocytes are found in damaged muscle only a very short time after damaging exercise (Fielding *et al.* 1993), largely due to the release of a number of inflammatory mediators (Section 2.5). It has also been suggested that superoxide derived from xanthine oxidase is involved in the attraction and activation of leucocytes (Granger 1988). This suggestion has been supported recently, as the areas of damaged muscle that showed the greatest xanthine oxidase immunoreactivity also had the greatest numbers of infiltrating leucocytes (Hellsten *et al.* 1997). Therefore, activated polymorphonuclear leucocytes may be responsible for considerable oxidative stress in the post-exercise period.

2.7 Effects of free-radicals

Free-radicals are potentially very toxic to cells, due to their highly reactive properties. Radicals react with many different molecules, depending on the reactivity of the radical species, and the susceptibility of the molecule to free-radical damage.

2.7.1 Lipid peroxidation

Unsaturated fatty acids are particularly susceptible to free-radical attack (Demopoulos 1973a). The presence of double bonds weakens the carbon-hydrogen bond of the carbon atom adjacent to the carbon with an unsaturated bond. These carbons are referred to as methylene carbons, and due to the weaker association with hydrogen, the hydrogen is more readily abstracted (Demopoulos 1973a). Therefore, the greater the number of unsaturated bonds, the greater the susceptibility to hydrogen abstraction. Figure 2.4 demonstrates the processes following exposure of unsaturated fatty acids to free-radicals. Following hydrogen abstraction (1), which leaves a lipid radical (2), there is bond rearrangement which produces a conjugated diene (3). Subsequently, the lipid reacts with oxygen to form a peroxy radical (4). The newly formed peroxy radical may

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abstract a hydrogen from another unsaturated fatty acid (5), forming a lipid hydroperoxide (6) and a further carbon-centred lipid radical (7). Therefore, once lipid peroxidation is initiated, it may theoretically continue in a cyclic fashion (8) until there are either no further unsaturated fatty acids available, or no oxygen to fuel the process. Lipid hydroperoxides are degraded to various aldehydes (9), principally malondialdehyde, and also hydrocarbons (10), ethane and pentane (Brown and Kelly 1996).
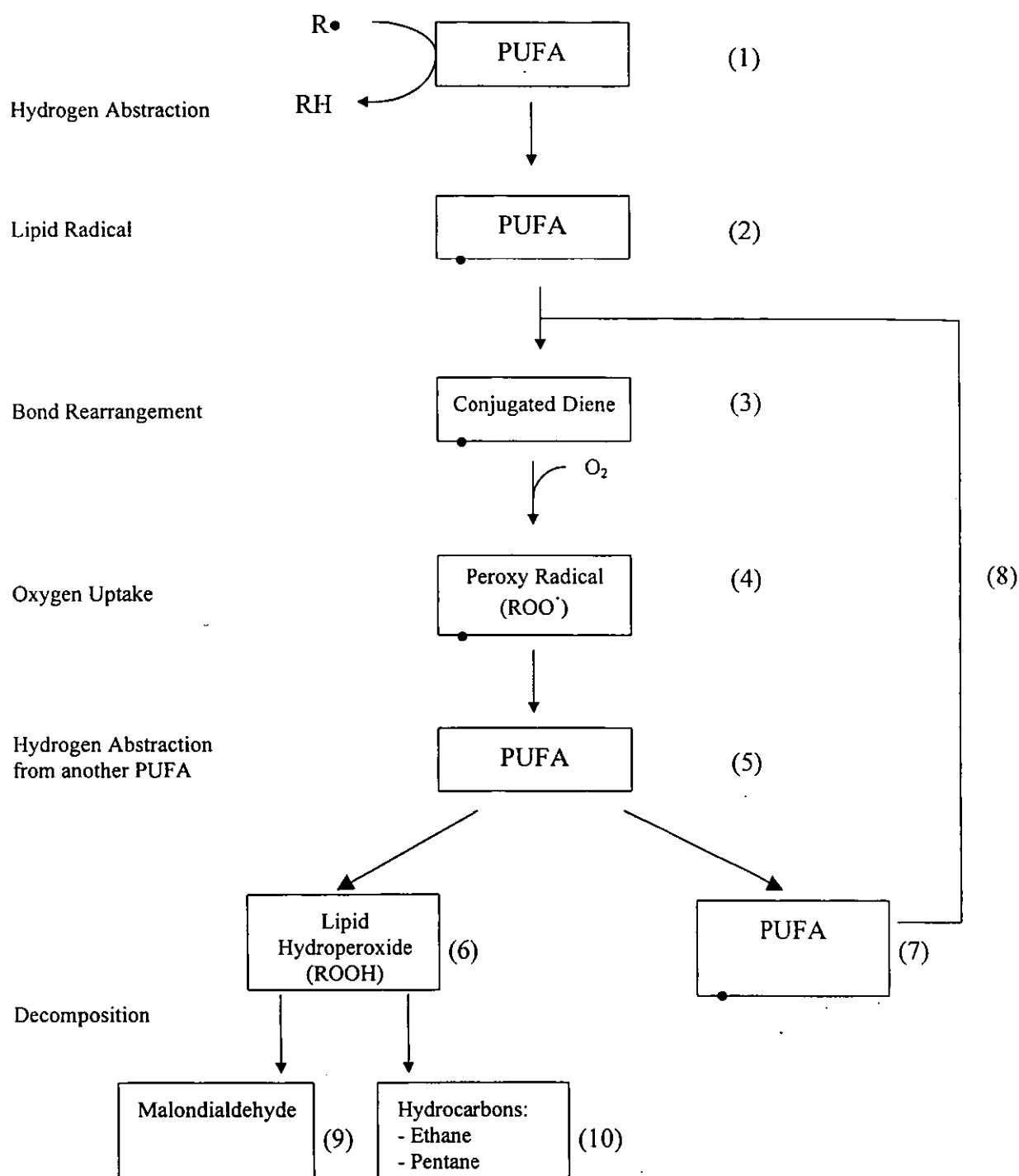
Figure 2.4   Lipid peroxidation.
Adapted from Rice-Evans (1990) and Gutteridge (1988). PUFA:
Polyunsaturated fatty acid. The dot signifies that the molecule is a
radical. The initial radical (R˙) may be OH˙, RO˙, ROO˙, or R˙.

Lipid hydroperoxides may also inititate new rounds of peroxidation due to the formation of peroxy and alkoxy radicals (Equations 6 and 7). Therefore, once lipid peroxidation is initiated, it may not only continue in a cyclic fashion, but may also become amplified. It is noteworthy that lipid peroxides may form rapidly and remain in place for prolonged periods of time, as long as days, before forming radical fragments (Demopoulos 1973a).

$$ROOH + Fe^{2+} \rightarrow Fe^{3+} + RO^{\cdot} + OH^{-} \tag{6}$$

$$ROOH + Fe^{3+} \rightarrow Fe^{2+} + ROO^{\cdot} + H^{+} \tag{7}$$

Polyunsaturated fatty acids are extremely prevalent in cell membranes, particularly those of organelles (Demopoulos 1973a), and are essential for maintaining membrane fluidity and function (Newsholme and Leech 1983). Therefore, lipid peroxidation may spread within the membrane and cause considerable damage, potentially leading to complete disruption of the membrane if allowed to proceed unchecked (Punchard and Kelly 1996). Alternatively, more minor peroxidation may simply affect membrane structure and function, largely due to a decrease in fluidity (Demopoulos 1973a). It is also possible that peroxidation of membrane unsaturated fatty acids leads to altered configuration of membrane bound proteins, and therefore impaired function (Jenkins 1988). Additionally, the disruption of membrane phospholipids may produce lysophospholipids, which are able to disrupt membranes due to their detergent-like properties (Newsholme and Leech 1983; Das and Engelman 1990). Furthermore, malondialdehyde is able to alter membrane structure, even in the absence of lipid peroxidation, possibly due to cross-linking within membranes (Pfafferott *et al.* 1982).

2.7.2 Protein damage

Proteins appear to aggregate and fragment upon exposure to a combination of superoxide and hydroxyl radicals (Davies 1987). Aggregation and fragmentation increases degradation, probably due to intracellular proteases (Wolff *et al.* 1986; Davies 1987). This may explain why even relatively minor damage to proteins may increase

rates of proteolysis (Wolff *et al.* 1986). The process by which this occurs seems similar to that for lipid peroxidation. Initially, there is hydrogen abstraction from an amino acid carbon atom, followed by reaction with oxygen to form a peroxy radical (Wolff *et al.* 1986). Subsequent decomposition of such peroxides would lead to protein fragmentation (Davies 1987), or alternatively, fragmentation may be due to the direct cleavage of certain side-chains (Wolff *et al.* 1986). Protein thiols are particularly susceptible to free-radicals (Demopoulos 1973b). The hydrogen of the sulphydryl (-SH) group is readily abstracted, forming a sulphur-centred radical (-S'). Sulphur-centred radicals typically react with other sulphur-centred radicals to form a non-reactive disulphide, which is the basis for the antioxidant quality of glutathione (Section 2.9.2).

Enzymes (Rice-Evans 1990), proteins in the respiratory chain (Cochrane *et al.* 1988), and ionic pumps (Kako 1987; Kim and Akera 1987) appear to be inactivated by free-radicals, and therefore alteration of protein structure has wide-reaching implications. It has been suggested that when large integrated proteins (e.g. myofibrillar proteins) are damaged, it may be some time before they are degraded, since they must be removed from structures prior to degradation (Wolff *et al.* 1986). Consequently, increased protein damage due to free-radicals has the potential to alter energy production, regulation of cell homeostasis, contractile function, and the normal function of certain enzymes. Damage to proteins may be initiated by the same free-radicals that initiate lipid peroxidation (OH', RO', ROO', R'). In particular, membrane bound proteins are susceptible to radicals generated as a result of neighbouring lipid peroxidation. Kramer *et al.* (1984) found a good relationship between malondialdehyde concentration and the inactivation of membrane sodium-potassium ATPase.

2.8 Free-radicals and Exercise

Exercise leads to increased free-radical production. The only method currently available for the direct measurement of free-radicals during exercise is electron spin resonance (ESR). This technique detects the weak magnetic field generated by the lone electron of a free-radical, whereas all other techniques measure the products of free-radical activity

(e.g. lipid peroxides). Early reports using this technique demonstrated that rat muscle homogenates showed a 2-3 fold increase in ESR signal after exhausting exercise (Davies *et al.* 1982) . Jackson *et al.* (1985) used ESR to study intact rat muscle following 30 min of stimulated contractile activity. There was a 70% increase in the ESR signal of freeze-clamped muscle following exercise, which was temporally related to increased activity of creatine kinase in the blood. A recent investigation applied ESR to exercising humans, using spin-trapping to produce stable free-radical adducts (Ashton *et al.* 1998). In this study, there was a three-fold increase in the ESR signal of human serum following a short bout of incremental cycling to exhaustion.

2.8.1 Indirect evidence of exercise-induced oxidative stress

The majority of studies which have assessed free-radical production following exercise have used indirect measurements, such as indices of lipid peroxidation. A very common approach has been to estimate malondialdehyde concentrations through the spectrophotometric determination of plasma thiobarbituric acid reactive substances (TBARS). Cycling at 70% $\dot{V}O_2$ max for 5 min has been shown to modestly increase TBARS, whereas cycling at 40% $\dot{V}O_2$ max for 5 min decreased TBARS below resting levels (Lovin *et al.* 1987). Furthermore, running for 30 min at 60% $\dot{V}O_2$ max increased TBARS above baseline, and increasing the intensity to 90% for 5 min increased TBARS further (Kanter *et al.* 1993). These studies suggest that there may be a relationship between TBARS and exercise intensity. Other studies have suggested that an increase in TBARS may be related to exercise-induced muscle damage. Demanding resistance exercise increased plasma TBARS, although not until 6-24h after exercise had finished, at a time when levels of creatine kinase were also elevated (McBride *et al.* 1998). This is supported by the observation that TBARS are increased 6 h after downhill running (Maughan *et al.* 1989), again at a time when CK values were also high. Additionally, Kanter *et al.* (1988) observed a correlation between CK and TBARS at the end of an 80km road race (r=0.62). Although this does not necessarily demonstrate a causal relationship, it does suggest a temporal association. Such a good relationship may initially appear surprising, since CK takes several hours to appear in the circulation (Volfinger *et al.* 1994), whereas MDA is cleared within a few hours

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(Jenkins 1993). One explanation for the correlation observed by Kanter *et al.* (1988) may be the long duration of exercise in this particular study (approximately 8.5h).

There are problems with the specificity of the TBARS assay (Brown and Kelly 1996), which have been largely overcome by the use of high-performance liquid chromatography (HPLC). Malondialdehyde (MDA) concentrations assessed by HPLC after exercise generally support the notion that exercise increases oxidative stress, although the values are much lower than using the TBARS assay. Ashton *et al.* (1998) showed an increase in MDA of 14% immediately after a cycling $\dot{V}O_2$ max test, and Child *et al.* (1998c) reported a 12% increase after 90 min of running.

Other techniques have also demonstrated that exercise produces oxidative stress. Expired hydrocarbons (pentane) are increased after cycling (Pincemail *et al.* 1990) and running (Kanter *et al.* 1993), apparently in relation to exercise intensity (Kanter *et al.* 1993). However, the use of this technique has potential problems, since hydrocarbons such as pentane are stored in tissue such as muscle (Jackson 1990). Recently, it has been reported that circulating levels of conjugated dienes increase after running 36km, but not after a 19km run (Vasankari *et al.* 1998). This suggests that exercise duration, in addition to exercise intensity, may be a factor in the initiation of the oxidative process. (Dufaux *et al.* 1997) used the ratio of reduced glutathione (GSH) to oxidised glutathione (GSSG) in whole blood as a marker of oxidative stress after a 2.5h run. This ratio fell to 18% of pre-exercise values after exercise, which was hypothesised to reflect increased reliance on the glutathione system. However, a potential problem with this method is that circulating levels of GSSG may increase due to release from muscle (Jackson *et al.* 1995), although this would not explain a fall in GSH.

There are also numerous reports that exercise has no effect on circulating markers of free-radical activity. Indeed, Sahlin *et al.* (1991) was unable to detect any malondialdehyde in plasma using HPLC, even after cycling at 100% $\dot{V}O_2$ max. Dufaux *et al.* (1997) also used HPLC, and reported no change in MDA after a 2.5h road race, although the ratio of GSH:GSSG fell after the same exercise. Running a marathon has also been shown to have no effect on immediate or 24h post-exercise plasma TBARS

(Rokitski *et al.* 1994a), and a half-marathon has been shown to have no effect on plasma TBARS or conjugated dienes (Duthie *et al.* 1990). It is likely that the failure to observe an increase in free-radical production is largely due to analytical problems, and the weight of the available evidence suggests that exercise leads to increased free-radical production.

2.8.2 Cell damage and free-radicals

Damage to cell membranes may be the single most important consequence of increased free-radical production during and after exercise. Lipid peroxidation or damage to membrane proteins would impair the regulation of homeostasis and lead to a rapid influx of calcium. Alternatively, intracellular levels of calcium may rise due to free-radical mediated damage to the sarcoplasmic reticulum (Hess *et al.* 1981; Hess et al. 1984). The consequences of elevated intracellular levels of calcium have already been discussed (Section 2.4.3), but include increased phospholipase activity, and the stimulation of calcium-activated proteases. Furthermore, both free-radicals and calcium are able to impair mitochondrial function (Malis and Bonventre 1988), which would threaten ATP resynthesis and further stress the cell's ability to maintain homeostasis. Cochrane *et al.* (1988) demonstrated that mitochondrial respiration is inhibited in a dose-dependent manner within a few minutes of incubation with hydrogen peroxide.

The temporal relationship between the many processes leading to cell damage was recently demonstrated in human tumour cells (Wu *et al.* 1996). Treatment of cells with a metabolic inhibitor led to a progressive increase in the levels of intracellular oxygen-centred radicals and calcium. Levels of both intracellular radicals and calcium peaked momentarily before membrane integrity was fully lost and cell death occurred. Although this experiment was not conducted in muscle cells, the sequence of events are probably very similar, and indeed may be enhanced in exercising muscle. Acidosis arising as a result of increased lactic acid formation during demanding exercise may increase the availability of iron for free-radical generation (Jenkins 1996). Furthermore, myoglobin is able to catalyse the formation of certain extremely damaging radicals (Mitsos *et al.* 1988). The involvement of iron in these processes has been demonstrated

by studies showing that iron chelators substantially decrease the extent of damage (Smith *et al.* 1989b).

Disturbed membrane function alone would lead to considerable areas of damage within cells, and possibly lead to cell death. However, radicals may also directly attack other cell components, and cause tissue damage independent to lipid peroxidation. Kong *et al.* (1994) used an inhibitor to prevent lipid peroxidation in rabbit myocardium, although this did not prevent contractile dysfunction. These authors suggested that this may be due to the oxidation of critical proteins, and that a reduction in lipid peroxidation may prevent necrosis in the absence of any benefit to contractile function. This form of damage has been termed 'stunning'. Although it reflects damage to cell components, it does not pose the same threat to skeletal muscle as damage to cell membranes. Contractile dysfunction in the myocardium of dogs may last up to 48h after ischaemia-reperfusion injury (Bolli *et al.* 1988). Since iron chelators substantially reduce the extent of dysfunction, it appears that radicals are partly responsible for this phenomenon (Bolli *et al.* 1990).

2.9 Protection against free-radicals

Before discussing the mechanisms which have evolved to offer protection against the effects of free-radicals, it is important to highlight that free-radicals also have potentially important beneficial roles. Without the ability to generate oxygen-centred radicals, phagocytes would have substantially reduced killing power (Pyne 1994; Smith 1994). Additionally, peroxidation is an important step in the biosynthesis of prostanoids and leukotrienes (Gutteridge 1988). Although this pathway has the potential to damage membranes (Duncan and Jackson 1987), the products of this process have numerous biological functions. Furthermore, limited and controlled release of reactive-oxygen species may modulate gene expression, and lead to the synthesis of proteins which offer protection and aid recovery from oxidative stress (Salo *et al.* 1991; Schulze-Osthoff *et al.* 1997; Jackson *et al.* 1998). In particular, the upregulation of heat-shock proteins is an attractive hypothesis for the explanation of decreased susceptibility to damage in

trained muscle (McArdle and Jackson 1996). Nevertheless, excessive production of free-radicals is undoubtedly detrimental, and the body has several levels of defence to cope with an acute increase in oxidative stress.

2.9.1 Antioxidant enzymes

There are a number of enzymes that have antioxidant properties (Equations 8-11), including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX). The hydroxyl radical is so reactive that it cannot be dealt with enzymatically, and therefore, selective enzymes have evolved to prevent its formation (Fridovich 1978). There are at least 2 forms of SOD, one found principally in the mitochondrial matrix, with manganese at the active site, and the other found mainly in the cytosol, with copper-zinc at the active site (Fridovich 1978). Catalase is found mostly in organelles such as peroxisomes, whereas glutathione peroxidase is found mainly in the cell cytosol. Glutathione peroxidase exists in two forms, one of which requires selenium (Sjödin *et al.* 1990). Both forms of glutathione peroxidase rely on two reduced glutathione molecules (GSH) donating a hydrogen from their sulphydryl groups to form oxidised glutathione (GSSG). Oxidised glutathione may be reduced by NADPH in a reaction catalysed by another enzyme, glutathione reductase. The NADPH for this process may be derived from increased activity of the hexose monophosphate shunt (Newsholme and Leech 1983).

$$O_2^{-} + O_2^{-} + 2H^{+} \xrightarrow{\text{SOD}} H_2O_2 \tag{8}$$

$$H_2O_2 + H_2O_2 \xrightarrow{\text{CAT}} 2H_2O + O_2 \tag{9}$$

$$H_2O_2 + 2GSH \xrightarrow{\text{GPX}} GSSG + 2H_2O \tag{10}$$

$$ROOH + 2GSH \xrightarrow{\text{GPX}} GSSG + ROH + H_2O \tag{11}$$
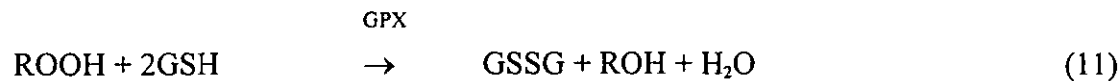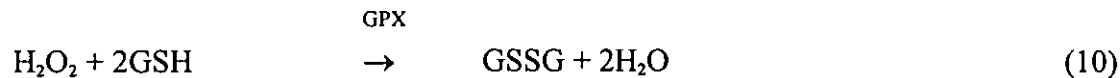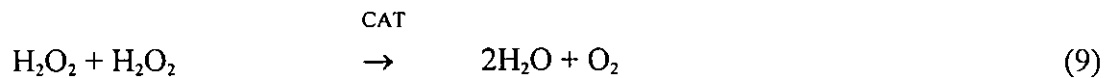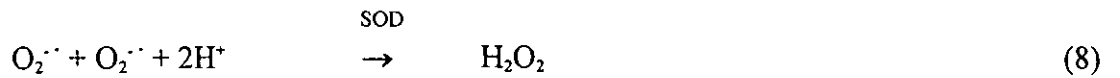
Removal of H_2O_2 and ROOH may be particularly important, since they are able to travel considerable distances, and initiate generation of more damaging radicals at new sites (see Equations 3,4,6,7) (Pryor 1986; Blake *et al.* 1987). These reactions rely on the presence of transition metals, and therefore systems have evolved to sequester metals by chelation. Molecules such as transferrin, lactoferrin, and haptoglobin sequester iron and haemoglobin, and ceruloplasmin and albumin sequester copper (Niki 1996).

Antioxidant enzymes are found principally inside cells, with only very low levels in extracellular compartments (Fridovich 1978; Del Maestro 1980). Therefore, it is likely that these enzymes play a major role in dealing with radicals produced by mitochondria, rather than extracellular sources of free-radicals. Endurance training increases the mitochondrial capacity of skeletal muscle (Brooks and Fahey 1985), and so a corresponding increase in the activity of antioxidant enzymes may be anticipated. Jenkins *et al.* (1984) proposed that this was the case, since subjects with a high $\dot{V}O_2$ max also had high levels of catalase and superoxide dismutase in the vastus lateralis muscle. Quintanilha (1984) showed increased activities of antioxidant enzymes in the muscles of endurance trained rats. However, not all antioxidant enzymes increase after training. Alessio and Goldfarb (1988) found that 18 weeks of training resulted in greater catalase activity in rats in response to an acute bout of exercise, although SOD was unaffected. Higuchi *et al.* (1985), using a demanding training schedule in rats, showed increased red and white muscle mitochondrial SOD, but not cytosolic SOD or catalase. A recent review on this topic suggested that, in general, training improves antioxidant enzymes by a large enough margin to offset exercise-induced oxidative stress (Powers *et al.* 1999).

2.9.2 Antioxidant compounds

There are a number of compounds present both inside and outside cells which have antioxidant properties. These include hydrophilic compounds such as vitamin C, uric acid, and proteins (e.g. glutathione); and hydrophobic antioxidants such as vitamin E, β-carotene, and ubiquinone (also known as Co-enzyme Q_{10}). Most of these represent chain-breaking antioxidants, since although they react with radicals and become

radicals in the process, they prevent the propagation of the free-radical chain since they are relatively stable (Bjørneboe *et al.* 1990; Niki 1996; Tsao 1997).

The low levels of antioxidant enzymes found outside cells has provoked interest into the level of antioxidant defences in extracellular compartments. Wayner *et al.* (1987) attempted to quantify the contribution of the major antioxidants in human plasma (Table 2.2). The total radical-trapping antioxidant parameter (TRAP) was calculated from estimated stochiometric radical trapping values for one molecule of each antioxidant. Although the total radical-trapping potential of plasma is undoubtedly a useful measure, calculating the contribution of each antioxidant poses several problems. The estimated TRAP that was based on stochiometric values was always much lower than the measured TRAP. These authors only measured protein thiols, and attributed the additional antioxidant protection to some other protein component, which explains the relatively high value for plasma proteins. A more recent study suggested that most of the antioxidant capacity of protein thiols was lost in auto-oxidation (Frei *et al.* 1989). Furthermore, using estimated stochiometric values for each antioxidant does not take into account any possible interaction or synergy between antioxidants. Finally, unless every antioxidant is accounted for, the contribution of certain components are likely to be overestimated. Wayner *et al.* (1987) also found ascorbate to be absent in the plasma of some subjects, which may suggest that analytical problems led to an underestimation of this component. Even after several weeks on a diet containing less than 5mg vitamin C d^{-1}, plasma concentrations of vitamin C are approximately 10 μmol.l^{-1} (Levine *et al.* 1996).

	Contribution to TRAP (%)	Range (%)
Uric acid	58	35 - 65
Plasma proteins	21	10 - 50
Vitamin C	14	0 - 24
Vitamin E	7	5 - 10

Table 2.2   Contribution of chain-breaking antioxidants to total radical-trapping antioxidant parameter (TRAP) in human plasma (Wayner *et al.* 1987).

In contrast to the findings reported in Table 2.2, other investigators have suggested that vitamin C is the most important antioxidant in plasma (Frei *et al.* 1989). Incubation of human plasma with a free-radical initiator showed that only vitamin C was able to completely prevent lipid peroxidation, whereas protein thiols, bilirubin, uric acid, and vitamin E could only delay the oxidative process. This study also highlighted that vitamin C preserved its antioxidant properties at very high concentrations (5 mmol.l^{-1}), although it was most efficient at low concentrations. This was attributed to some auto-oxidation at high levels, although these were well above physiological concentrations. Therefore, when radicals are generated or released in the extracellular compartment, vitamin C may be the only antioxidant capable of preventing the initiation of radical chain-reactions.

Plasma levels of antioxidants do not reflect the relative importance of each antioxidant within cells. The majority of cellular antioxidants operate by preventing radical propagation, and are active in the cytosol. These include antioxidant enzymes (SOD and GPX), and antioxidant compounds (vitamin C and GSH). It is possible that the optimal strategy is to quench radicals before they react with lipids and other cell components (Frei *et al.* 1989). Once lipid peroxidation has been initiated, it is largely the responsibility of vitamin E to scavenge radicals. Vitamin E has been described as the last line of defence against membrane peroxidation (Niki 1987), and therefore has an important role. Although there is only one molecule of vitamin E for approximately 2100 molecules of PUFA in mitochondrial membranes (Gruger and Tappel 1971), vitamin E scavenges approximately 90% of peroxy radicals before they attack lipids (Niki 1996). This is largely a reflection of the efficiency of vitamin E, which scavenges peroxy radicals about 10 times faster than lipids react with radicals (Niki 1996). However, the capacity of vitamin E would be very quickly exhausted if there was no system for recycling vitamin E from the vitamin E radical. This is achieved through the interaction of hydrophobic and hydrophilic antioxidants in different compartments. This was first suggested for vitamins E and C (Packer *et al.* 1979; Niki 1987), although it has now been extended to incorporate other cellular antioxidants (Figure 2.5).
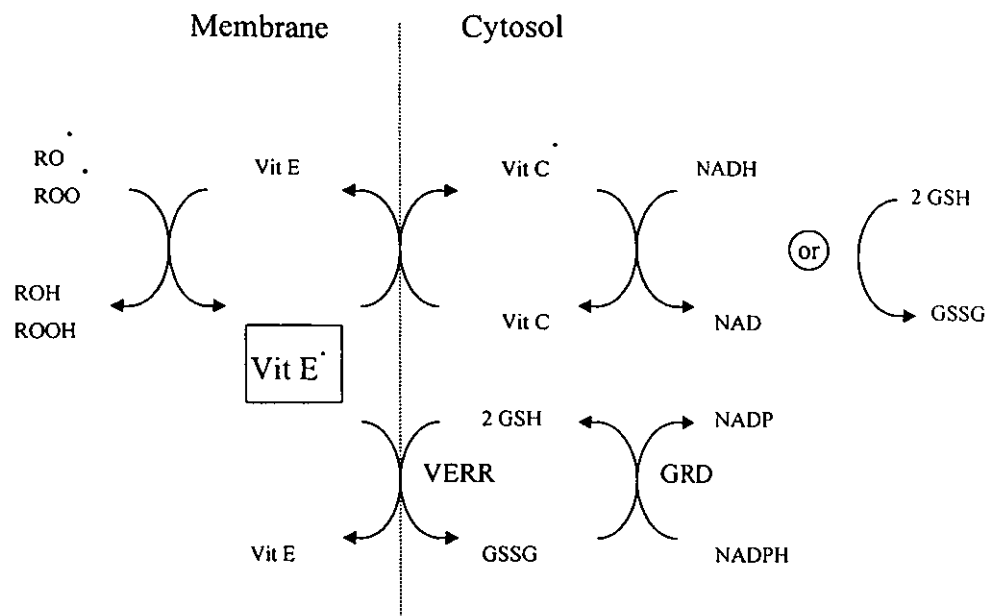
Figure 2.5   The relationship between some antioxidant compounds at the membrane-cytosol interface. Adapted from Chan (1993). VERR: vitamin E radical (tocopheroxyl) reductase. GRD: Glutathione reductase

It is this interaction with vitamin E that allows vitamin C and GSH to scavenge peroxy radicals that are within cell membranes. Figure 2.5 shows the single electron oxidation of vitamin C (ascorbic acid), which forms the vitamin C radical (semidehydroascorbyl). This is reduced to ascorbic acid by NADH, which may partly rely on the action of semidehydroascorbate reductase (Chan 1993). Further oxidation of semidehydroascorbyl forms dehydroascorbic acid (DHAA), which may irreversibly decompose to diketogluconic acid. Alternatively, DHAA may be reduced to ascorbic acid in a glutathione-dependent reaction. Reduction of DHAA may proceed independently, or may be catalysed by dehydroascorbate reductase. The role and existence of many of these systems *in vivo* is yet to be fully determined, although it is clear that there is considerable interaction between these antioxidants in solution (Packer *et al.* 1979), and in platelet homogenates (Chan 1993). Importantly, the recycling of the vitamin E radical by GSH is an enzymatic process, and is therefore considerably slower than the rapid non-enzymatic action of vitamin C (Chan 1993). Therefore, both GSH and vitamin C are able to regenerate vitamin E, although in situations where radical formation is rapid, vitamin C may play a more important role.

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It is also noteworthy that according to this system, the scavenging of potentially damaging free-radicals is ultimately linked to NADH and NADPH oxidation, and therefore normal metabolism.

2.10 Free-radical scavenging and antioxidants

Free-radicals clearly have the potential to damage cellular structures, and certain forms of exercise have been shown to increase oxidative stress. Nevertheless, this relationship does not demonstrate that free-radicals are responsible for exercise-induced muscle damage and soreness. Indeed, the situation may theoretically be reversed, as damage to tissues almost certainly activates certain free-radical processes (Demopoulos 1973b; Gutteridge 1988). It is not clear whether such activation would subsequently produce more widespread damage, although this is an attractive additional explanation for the post-exercise amplification of damage discussed in Section 2.5.4. Although a great deal of evidence has accumulated over the years, it has not been clearly demonstrated that increased free-radical production is associated with muscle damage and soreness. The greatest evidence that free-radicals are involved in muscle damage stems from studies which have shown that either preventing radical formation, or preventing them reacting with cellular components, offers some degree of protection.

2.10.1 Allopurinol and superoxide dismutase

Inhibition of xanthine oxidase by the administration of allopurinol has been shown to reduce the amount of damage in dog myocardium after ischaemia followed by reperfusion (Chambers *et al.* 1985). Additionally, injection of allopurinol appears to reduce the extent of lipid peroxidation in rat liver after exhaustive exercise (Koyama *et al.* 1999). However, Bushell *et al.* (1996) found that continuous infusion of allopurinol during prolonged ischaemia of rabbit skeletal muscle had no effect on the extent of ultrastructural damage after reperfusion. It is possible that there may be a delayed beneficial effect, as allopurinol administered for several days prior to demanding running in mice had no effect on ultrastructural damage immediately after exercise, but led to a marked reduction in skeletal muscle ultrastructural damage over the following

few days (Duarte *et al.* 1993). This may suggest that much of the damage that takes place in the post-exercise period is linked to increased activity of xanthine oxidase.

There are reports that treatment with superoxide dismutase protects against the effects of ischaemia followed by reperfusion in dog myocardium (Chambers *et al.* 1985). Similar beneficial effects have been seen in skeletal muscle after exercise. Injection of SOD prior to a 70 min run decreased plasma and muscle TBARS up to 3 days after exercise in rats (Radak *et al.* 1995). Mouse muscle exposed to a damaging eccentric exercise protocol also showed significantly less ultrastructural damage in the few days after exercise following treatment with SOD (Zerba *et al.* 1990b). This study also provided support for the notion that damage during exercise is partly due to the effects of free radicals, as old mice treated with SOD showed less damage than age-matched controls immediately after exercise. It is noteworthy that the effects of SOD may be partly due to the inhibition of xanthine oxidase (Radak *et al.* 1995), in addition to the direct scavenging of superoxide.

An interesting study recently used a pollen extract with similar properties to SOD to investigate responses to damaging exercise in humans (Krotkiewski *et al.* 1994). After 4 weeks of supplementation, subjects performed a number of demanding forms of exercise, that alternated between bench stepping and cycling, and lasted almost 3h. Treatment with pollen extract reduced plasma and muscle MDA, the serum activity of creatine kinase, and soreness. However, there was no difference in the maximal force of the knee extensors between supplemented and placebo groups.

2.10.2 *Vitamin E*

Vitamin E is the term used to describe 8 naturally occurring essential fat-soluble nutrients (4 tocopherols and 4 tocotrientols). It appears that α-tocopherol has the highest biological activity, and is the most prevalent in foods and body tissues (Bjørneboe *et al.* 1990; Meydani *et al.* 1993). The major biological function of vitamin E revolves around its ability to act as a lipid soluble antioxidant (Niki 1996). Much of the vitamin E in human diets comes from flour and vegetable oils, and is absorbed in

the small intestine in conjunction with fat (Bjørneboe *et al.* 1990). Vitamin E is transported in the blood almost entirely via the lipoproteins (Behrens *et al.* 1982).

Vitamin E is not only found in the plasma membrane, but also in mitochondria, microsomes, lysosomes, and the golgi apparatus (Bjørneboe *et al.* 1990). Mitochondrial membrane vitamin E may play a vital role in dealing with electrons that escape during normal respiration. Vitamin E deficient rats have approximately 40% decreased endurance capacity (Davies *et al.* 1982; Gohil *et al.* 1986). These authors reported that the $\dot{V}O_2$ max values of the two groups were similar, and suggested that decreased endurance reflected the inability to maintain ATP resynthesis due to mitochondrial membrane damage. Vitamin E, in conjunction with other antioxidants, has considerable ability to scavenge many different radical species (Section 2.9.2). Animal studies have demonstrated that diets deficient in vitamin E are associated with increased lipid peroxidation. Seven weeks on a vitamin E deficient diet increased pentane and ethane production in proportion to the length of time that rats had been deficient (Dillard *et al.* 1977). Furthermore, 5 weeks of deficiency in resting mice increased skeletal muscle lipid peroxidation, and the susceptibility to iron-catalysed lipid peroxidation (Salminen *et al.* 1984).

Skeletal muscle vitamin E concentrations have been shown to decline after a period of prolonged training (Aikawa *et al.* 1984), and after an acute bout of exercise in rats (Bowles *et al.* 1991). It appears that it may take 72h for muscle levels to be replenished after an acute bout of demanding exercise (Swift *et al.* 1998). Additionally, although endurance training has been shown to increase the ubiquinone content of rat muscle, there does not appear to be a corresponding increase in vitamin E (Gohil *et al.* 1987; Packer *et al.* 1989). These authors suggested that the amount of vitamin E may be diminished, in relation to training induced alterations in mitochondrial capacity. Furthermore, 8 weeks of training in vitamin E deficient rats does not lead to higher levels of antioxidant enzymes, in order to account for the poorer antioxidant protection in the absence of this vitamin (Tiidus and Houston 1994).

Liver, skeletal muscle, adipose tissue, and plasma have the capacity to accumulate vitamin E (Bjørneboe *et al.* 1990). Prolonged supplementation with α-tocopherol increased skeletal muscle and plasma levels by 31% and 124%, respectively (Meydani *et al.* 1993). Such an increase may occur at the expense of other less biologically active forms of vitamin E, as this study also reported that γ-tocopherol levels fell over the same period. Early animal studies suggested that adding vitamin E to the diet of rats offered substantial benefits (Brady *et al.* 1979; Jackson *et al.* 1983). However, as Table 2.3 highlights, this may have been partly due to a degree of deficiency in the control animals. Nevertheless, more recent studies in animals and humans suggest that supplementation with vitamin E above control levels may reduce oxidative stress and markers of muscle damage after certain forms of exercise (Tables 2.3 and 2.4). Although many studies report favourable changes following supplementation, some animal and human investigations have observed no benefits from the provision of additional vitamin E (Warren *et al.* 1992; Jakeman and Maxwell 1993).

Animal studies	Animals	Supplement	Exercise	Outcome	Notes
Brady *et al.* (1979)	Rats	50IU/kg food for 4 weeks	Swimming to exhaustion (10-15min)	↓ liver TBARS ● muscle TBARS	Control animals received no vitamin E in diet
Jackson *et al.* (1983)	Rats	360 IU/kg food for 6 weeks	Electrical stimulation (30min)	↓ serum CK ● muscle TBARS	Control animals received no vitamin E in diet
Kumar *et al.* (1992)	Rats	220 IU/kg food for 60 days	Swimming (30min)	↓ cardiac muscle MDA at rest and after exercise ↓ cardiac muscle ESR signal after exercise	
Reznick *et al.* (1992)	Rats	10000 IU/kg food for 4 weeks	Treadmill running (60-90min)	↓ muscle protein oxidation at rest and after exercise	
Warren *et al.* (1992)	Rats	10000 IU/kg food for 5 weeks	Downhill walking (150min)	● ultrastructural damage ● serum CK ● Force	Followed 0-48h post-exercise
Van Der Meulen *et al.* (1997)	Rats	Injected over 5 days*	Electrical stimulation Eccentric (225 actions)	● ultrastructural damage ↓ serum CK ● Force	Followed 0-72h post-exercise

Table 2.3   Vitamin E supplementation in animals.
↓ This parameter reduced in comparison to control animals. ● No difference between treated and control animals.
* Produces similar muscle levels to 5 weeks of supplementation with high doses of vitamin E.

Human Studies	Subjects	Supplement	Exercise	Outcome	Notes
Dillard *et al.* (1978)	Males and females	1200IU/d for 2 weeks	Cycling at 50% $\dot{V}O_2$ max	↓ pentane at rest and during exercise	
Simon-Schnass and Pabst (1988)	Not specified	600IU/d for 4 weeks	Mountain climbing	↓ pentane	Resting measurements determined at 5000m
Sumida *et al.* (1989)	Males	450IU/d for 4 weeks	Incremental cycling to exhaustion (~19min)	↓ serum TBARS	
Jakeman and Maxwell (1993)	Males and females	600IU/d for 21d	Bench stepping (60min)	• serum CK • Muscle function	Continued supplementation for 7d post-exercise
Meydani *et al.* (1993)	Males	800IU/d for 48d	Downhill running (45min)	↓ Urinary TBARS	Only different 12d post-exercise
McBride *et al.* (1998)	Males	1200IU/d for 2 weeks	Resistance exercise	↓ serum CK ↓ plasma TBARS	TBARS lower post-exercise, but higher immediately after exercise in supplemented group

Table 2.4   Vitamin E supplementation in humans.
↓ This parameter reduced in comparison to a control group. • No difference between treated and control groups.

It is noteworthy that vitamin E does not appear to influence either ultrastructural damage or muscle function after exercise (Tables 2.3 and 2.4), only indices of membrane damage and permeability (e.g. TBARS and CK). This may suggest that preserving membrane integrity alone has no impact on intracellular parameters. It should also be pointed out that the benefits of vitamin E cannot be solely attributed to its antioxidant functions, since it also stabilises membranes through direct interaction with phospholipids (Kagan *et al.* 1989; Niki 1996).

2.10.3 Vitamin C

As already discussed in Section 2.9.2, vitamin C may exist in both a reduced (ascorbic acid) and oxidised (dehydroascorbic acid) form. In most mammals, ascorbic acid is not classed as a vitamin, because their livers have the capacity to synthesise ascorbic acid from glucose (Buettner and Jurkiewicz 1996). Primates and guinea pigs, however, rely on sufficient absorption from the diet in order to acquire and maintain elevated levels of vitamin C (Brown and Jones 1996).

2.10.3.1 Vitamin C absorption and tissue levels

The majority of vitamin C in foods (80-90%) is in the form of ascorbic acid (Rumsey and Levine 1998). Vitamin C is absorbed in the small intestine in a sodium-dependent active transport process (Rose 1996). Absorption of vitamin C is not affected by the dietary form in which it is provided, as broccoli, orange juice, fruit, and synthetic vitamin C all increased plasma levels similarly (Mangels *et al.* 1993). It appears that ingestion of more than 3g of vitamin C saturates the capacity of intestinal absorption, and leads to plasma levels of $200\mu mol.l^{-1}$ approximately 3h after taking a one-off dose (Hornig *et al.* 1980). Dividing the dose of vitamin C into several smaller doses, or providing it with a meal, may increase the amount that is absorbed by approximately 60% (Yung *et al.* 1981). Nevertheless, ingestion of large amounts of vitamin C will only transiently increase circulating levels. Hornig *et al.* (1980) reported that 75% of a 1g dose was absorbed and retained, whereas this is the case for only 20% of a 5g dose. This appears to be due to the fact that once plasma levels increase above approximately $65\mu mol.l^{-1}$, reabsorption in the kidney is saturated, and renal excretion is increased

(Brown and Jones 1996). Consequently, because humans have a limited capacity for both absorption and retention of vitamin C, it seems very unlikely that overload can occur (Hornig and Moser 1981). However, by the same token, it suggests that it is difficult to achieve very high circulating levels, unless vitamin C is consumed on a regular basis.

It has been suggested that prolonged high intakes of vitamin C may reduce levels of absorption (Rose 1996). According to this hypothesis, if dietary intake of vitamin C was suddenly reduced, or an individual stopped taking a supplement, the level of vitamin C in the body would fall until absorption was upregulated. This potential phenomenon has been termed 'rebound scurvy'. Karasov et al. (1991) showed that the small intestine of guinea pigs previously fed a diet rich in vitamin C exhibited a dramatic decline in the ability to absorb vitamin C. It was suggested that prolonged ingestion of high levels of vitamin C had led to the downregulation of absorption. However, normal levels of absorption were restored within 1-2 weeks (Karasov et al. 1991), and therefore it is unlikely that a deficiency of any kind could develop in such a short space of time.

Total body stores of vitamin C in humans have been estimated to be between 1.5 and 5g (Baker 1967; Combs 1992). The tissue distribution of vitamin C in typical human tissues is shown in Table 2.5. It has been suggested that plasma levels of vitamin C reflect recent dietary intake, and are not a good indicator of vitamin C status, unless intake has been constant for several days (Jacob et al. 1987). A more useful measure of vitamin C status may be leucocyte vitamin C content, as this declines more slowly in the event of dietary vitamin C restriction, and may give an indication of tissue levels (Albanese et al. 1975; Jacob et al. 1987). However, there are drawbacks in simply measuring total leucocyte vitamin C, since the separation procedures employed may lead to interference from platelet vitamin C. Evans et al. (1982) suggested that platelets may contribute up to 80% of the vitamin C attributed to leucocytes, and therefore it is advisable to assess vitamin C in individual types of leucocyte. Evans et al. (1982) estimated that in human blood, 38% of vitamin C is in the plasma, 36% in erythrocytes, 16% in platelets, 6% in mononuclear cells, and 4% in polymorphonuclear leucocytes.

Tissue	Vitamin C (mg/kg)	Percentage of Body fraction*	Tissue Vitamin C (mg)	Percentage of total vitamin C
Skeletal Muscle	35	43.0	1050	52
Brain	140	2.3	225	11
Liver	125	2.0	175	9
Skin	30	7.0	147	7
Adipose Tissue	10	20.0	140	7
Lungs	70	1.6	79	4
Blood	9	8.0	49	2
Kidneys	55	0.5	19	1
Heart	55	0.4	15	1

Table 2.5   Approximate tissue concentrations and body stores of vitamin C. Values for a 70kg individual with total body stores of 2g. * Percentage of body fraction expressed as organ weight per kg body weight. Taken from Brown and Jones (1996).

2.10.3.2 Transport of vitamin C

The process by which certain cells accumulate vitamin C against a steep concentration gradient is unclear. Cells such as lymphocytes are able to accumulate vitamin C concentrations more than 50 times greater than the plasma (Tsao 1997). Furthermore, certain cells appear to be able to accumulate vitamin C at specific times. Activation of neutrophils leads to a rapid increase in the rate of vitamin C uptake, possibly as a mechanism for protection from self-induced oxidative damage (Washko *et al.* 1993). Other cells, such as erythrocytes, simply accumulate vitamin C in proportion to the amount in the plasma (Evans *et al.* 1982).

It has been suggested that vitamin C is transported into cells exclusively as dehydroascorbic acid (DHAA), and is rapidly reduced to ascorbic acid (AA) internally (Vera *et al.* 1993; Washko *et al.* 1993; Agus *et al.* 1997). However, it seems more

likely that AA is also transported, but at much lower rates than DHAA (May *et al.* 1995; Rumsey and Levine 1998). Such a selective system allows the maintenance of high intracellular concentrations of vitamin C, as once it is accumulated in its reduced form, it cannot readily escape. The cells which have been studied to date appear to transport DHAA largely by the glucose transporters (Vera *et al.* 1993; May *et al.* 1995). The uptake and accumulation of vitamin C in skeletal muscle is unclear at the present time, although smooth muscle cells also appear to utilise the glucose transporters (Voskoboinik *et al.* 1998). The rate of transport of DHAA by GLUT 1 and GLUT 3 is similar to their rate of glucose transport, whereas GLUT 2 and GLUT 5 are incapable of transporting DHAA (Rumsey *et al.* 1997). Although, GLUT 4 appears to transport DHAA, the rate is much lower than the rate of glucose transport (Rumsey *et al.* 1997). The prevalence of GLUT 1 and GLUT 4 transporters in skeletal muscle (Ivy and Kuo 1998), may mean that they play a significant role in vitamin C uptake in these tissues. Glucose inhibits the uptake of vitamin C in oocytes (Vera *et al.* 1993) and in the brain (Agus *et al.* 1997), although not in erythrocytes (Himmelreich *et al.* 1998) or osteoblasts (Pandipati *et al.* 1998). The process by which DHAA is reduced once inside cells is not fully understood. It has been proposed that DHAA may be reduced to AA by reduced glutathione, with oxidised glutathione being reduced by NADPH derived from the hexose monophosphate shunt (Washko *et al.* 1993).

The availability of DHAA would appear to be a prerequisite for considerable cellular uptake and accumulation of vitamin C, although it is AA which has the greatest biological activity. Concentrations of DHAA up to 29 μmol.l^{-1} have been reported in the plasma of normal individuals (Nagy and Degrell 1989; Sinclair *et al.* 1991). Consequently, the ratio of DHAA:AA in plasma may theoretically be a useful index of oxidative stress, in a similar manner to the ratio of GSH:GSSG. There is some support for this notion, as diabetics and individuals with rheumatoid arthritis appear to have a higher ratio than people without these conditions (Lunec and Blake 1985; Sinclair *et al.* 1991). A recent study, however, suggested that DHAA is not present in human plasma (Dhariwal *et al.* 1991). These authors provided strong evidence to show that if DHAA is present, it is less than 0.5% of total vitamin C. This finding was supported with a separate study showing that if plasma samples were left standing in refrigerated

conditions, there was a progressive increase in DHAA in proportion to time (Dhariwal *et al.* 1991). This may suggest that reports of DHAA in plasma are artefactual, since the methods used in the studies mentioned earlier rely on a prolonged incubation period (Lunec and Blake, 1985; Sinclair et al. 1991). Very low levels of DHAA may not be too surprising, since cells would rapidly take up and remove DHAA if it were to become available. Furthermore, DHAA is extremely unstable at physiological temperatures and pH, and would rapidly be irreversibly converted to diketogulonic acid (Lunec and Blake 1985).

2.10.3.3 Vitamin C requirements

Catabolism of vitamin C in an average person has been suggested to be close to 22mg per day (Baker 1967). Other estimates range from 34-60mg per day, and are based on the fact that with an estimated body store of 1.5g, signs of scurvy develop after approximately 30 days (Levine 1986). This is the rationale for the current reference nutrient intake (RNI) of 40mg (DHSS 1991), as this amount maintains body stores in the majority of the population, and therefore prevents deficiency and scurvy. However, it has been suggested that the amount that is required to prevent deficiency, and the amount which would be most beneficial to biochemical reactions, may be very different (Levine 1986). Furthermore, the requirements for vitamin C increase with increasing stress, including physical stress (Baker 1967). Consumption of 60mg of vitamin C amounts to approximately 0.9mg per kg body mass in an average person. It has been suggested that humans would benefit from the consumption of much higher levels of vitamin C, which may reflect the amounts synthesised in animals that are able to do so (Levine 1986). Rates of synthesis range from 5 mg kg^{-1} d^{-1} for cats and dogs, up to more than 200 mg kg^{-1} d^{-1} in rats and mice (Levine 1986).

Jacob *et al.* (1987) suggested that daily consumption of 138mg of vitamin C would be an appropriate intake, as this amount would saturate the body pool. In a recent very well-controlled study, subjects were housed in a metabolic ward for several weeks, and received various amounts of vitamin C in their diets (Levine *et al.* 1996). The overall results of this study suggested that an appropriate RDA would be 200mg per day. This

conclusion was based on the fact that at this dose, bioavailability was 100%, and leucocyte levels (neutrophils, monocytes and lymphocytes) appeared to be fully saturated. It has been estimated that ingestion of this dose (200mg a day) would increase the total body pool from 1.5 to 3g (Levine 1986). Ingestion of more than this amount did not appear to increase leucocyte levels any further, although plasma levels were not fully saturated until a daily dose of 1g was consumed (Levine *et al*. 1996).

2.10.3.4 Functions of vitamin C

Vitamin C has a wide range of functions throughout the body, in addition to the antioxidant properties already discussed in Section 2.9.2. Collagen synthesis relies on vitamin C, and therefore so too does the maintenance of connective tissue. Vitamin C is a co-factor in the hydroxylation of the amino acids proline and lysine, which forms hydroxyproline (an essential element of collagen). It is the ability of vitamin C to reduce iron at the centre of the enzymes which catalyse this reaction which makes hydroxylation possible (Tsao 1997). Vitamin C is also required for the synthesis of certain hormones (e.g. noradrenaline, vasopressin and cortisol) (Levine 1986), and may protect hormones against oxidation once in the circulation (Tsao 1997). Additionally, vitamin C appears to be essential for the normal synthesis of carnitine, which has been speculated to partly explain the lethargy and weakness associated with scurvy (Burri and Jacob 1997). Vitamin C also increases iron and selenium absorption in the intestine, while decreasing copper, nickel, and manganese absorption (Tsao 1997). There are many other functions of vitamin C, but most appear to revolve around its ability to act as a reducing agent. Importantly, although other reductants may replace vitamin C in many of these reactions, it has been suggested that maximal activity is achieved only by the presence of vitamin C (Levine 1986).

2.10.3.5 Supplementation with vitamin C and exercise

Over the years, a variety of beneficial claims have been made regarding supplementation with vitamin C in active people. Many of these have no firm scientific basis, or have been derived from poorly controlled studies. Syed (1966) claimed that soreness after exercise could be prevented by taking 500mg of vitamin C before

exercise and 400mg after exercise. Furthermore, if soreness did appear after this regimen, it could be 'easily cleared by taking 400mg of extra vitamin C and extra fluids' (Syed 1966). Early experimental attempts to confirm this proved unsuccessful, although there were problems with study design due to a repeated-bout effect on the second exercise test (Corbett 1967).

It appears that supplementation with 1g of vitamin C a day for 5 days, which increased whole-blood vitamin C from 46 μmol.l^{-1} to 90 μmol.l^{-1}, may improve mechanical efficiency during cycling (Hoogerwerf and Hoitink 1963). Additionally, electrical stimulation of muscle taken from rats supplemented with vitamin C took longer to fatigue than equivalent muscle from control rats (Richardson and Allen 1983). It is probably a direct result of such purported benefits that led to one of the more striking claims regarding vitamin C, that the outstanding Russian performance during the 1960 Olympic games was attributable to supplementation with this vitamin (Van Huss 1966). Although reports of an improvement to metabolism and performance following vitamin C supplementation remain largely unsubstantiated, there may be a physiological mechanism to explain these findings. Buzina and Suboticanec (1985) reported that vitamin C deficient boys had low $\dot{V}O_2$ max values, which increased following supplementation. These authors highlighted that increasing plasma status above this level had no further benefit, but that in vitamin C deficient individuals, supplementation was worthwhile. The importance of adequate vitamin C intake in active people has been supported recently. Johnston *et al.* (1999) reported that mechanical efficiency during exercise increased after depleted subjects were repleted with 500mg vitamin C a day, although efficiency increased by only 1%.

There is no evidence, however, that individuals with reasonable vitamin C status would improve performance through the ingestion of higher levels of vitamin C. Interestingly, Packer *et al.* (1986) showed that both vitamin C deficiency and supplementation may be detrimental. Guinea pigs were placed on normal, deficient, and supplemented diets for 8-10 weeks prior to exhausting exercise. Animals on a normal diet exercised for longer than either deficient or supplemented animals, and the authors suggested that there may be an optimal level of dietary vitamin C in animals unable to synthesise this

vitamin. This appears to suggest that although it is important to avoid deficiency, it is equally important to avoid excessive intakes. In another study using guinea pigs, supplementation with vitamin C could not offset impaired exercise capacity in vitamin E deficient animals (Gohil et al. 1986).

Vitamin C has been claimed to influence the severity of the common cold for more than 70 years (Hemila 1997a). Since it appears that demanding exercise may increase the susceptibility to infection (Peters 1997), vitamin C may be particularly important in active people. A recent analysis of studies which had investigated the effect of vitamin C supplementation and the incidence of common colds in active individuals suggested that there may be a moderate beneficial effect (Hemila 1996). However, the mechanism for such an effect, if it exists, remains unclear. Hemila (1997b) suggested that vitamin C had many effects on the immune system, and that it was likely that no single mechanism was activated by increased intakes of vitamin C. It has been proposed that a major role of vitamin C is to degrade histamine, which if present for prolonged periods may lead to immunosuppression (Johnston et al. 1992b). Analysis of 437 human blood samples suggested that once plasma levels of vitamin C fall below 57 μmol.l^{-1}, concentrations of histamine increase proportionally (Clemetson 1980). Furthermore, prolonged supplementation with vitamin C, but not acute supplementation, has been shown to reduce plasma levels of histamine in non-active asymptomatic people (Johnston et al. 1992b).

It is noteworthy that well-trained individuals exhibit better plasma and cellular vitamin C status than their sedentary counterparts (Fishbaine and Butterfield 1984; Robertson et al. 1991). This may suggest a training induced adaptive response, possibly a reduction of renal excretion in more active individuals (Fishbaine and Butterfield 1984). Alternatively, it may simply reflect the fact that active individuals appear to consume more vitamin C than their less-active counterparts (Fogelholm 1994; Rokitski et al. 1994b). However, a physiological mechanism whereby a greater level of vitamin C is accumulated in active individuals remains an attractive hypothesis, which may have potential benefits. Gleeson et al. (1987) reported that plasma levels of vitamin C fell the day after a demanding 21km road race, and therefore elevated initial concentrations

may be advantageous. Interestingly, although plasma levels of vitamin C fall in the long term after physical stress (Baker 1967; Shukla 1969; Hume *et al.* 1972; Gleeson *et al.* 1987), the acute effect of exercise is to increase circulating levels of vitamin C (Gleeson *et al.* 1987; Duthie *et al.* 1990; Maxwell *et al.* 1993; Rokitski *et al.* 1994a). It has been proposed that the adrenal gland is the major source of increased levels of circulating vitamin C during exercise, and a good relationship between the increase in cortisol and the increase in plasma vitamin C has been observed (Gleeson *et al.* 1987).

Although speculative, it is possible that increased plasma levels of vitamin C during exercise reflect a mechanism to protect against exercise-induced free-radical production. Increased free-radical degradation of vitamin C may also explain the fall in circulating levels of vitamin C observed after a single bout of demanding exercise (Gleeson *et al.* 1987). Therefore, the requirement for vitamin C may be increased in active individuals. Furthermore, the potential role of radicals in the aetiology of muscle damage may mean that vitamin C is in the greatest demand during or after damaging exercise (Sections 2.6-2.8). Consequently, increasing the availability of vitamin C at a time of exercise-induced oxidative stress may theoretically diminish subsequent muscle damage and soreness. In spite of this potential benefit, information on exercise-induced oxidative stress and vitamin C is scarce, which Sen (1995) recently described as 'remarkable'. Furthermore, Goldfarb (1999) recently provided the following point of view; 'Clearly there is a paucity of good research on the effectiveness of dietary vitamin C supplementation in influencing muscle damage and soreness'. Goldfarb (1999) reached this conclusion after the failure to find more than one or two well-controlled investigations on this topic. The few relevant investigations that have been carried out are included in Table 2.5. Vitamin C has been shown to have beneficial effects on exercise-induced muscle soreness (Kaminski and Boal 1992), lipid peroxidation (Vasankari *et al.* 1998), and muscle function (Jakeman and Maxwell 1993). Furthermore, a recent study in rabbits showed that injury induced by ischaemia-reperfusion was drastically reduced when muscles were infused with vitamin C (Bushell *et al.* 1996). Notably, vitamin C infusion decreased ultrastructural damage, the efflux of CK, and the concentration of myeloperoxidase in affected muscle.

Study	Subjects	Supplementation	Exercise	Outcome	Notes
Staton (1952)	Males	100mg per day for 30d	3min of sit-ups	• Performance 24h post-exercise	Postulated that lower performance is a measure of soreness
Kaminski and Boal (1992)	Males and females	3000mg per day for 3d	One-legged body lifting and lowering (plantar- and dorsiflexion of one foot)	↓ soreness	Continued supplementation for 7d post-exercise
Jakeman and Maxwell (1993)	Males and females	400mg per day for 21d	Bench Stepping	• serum CK ↑ Muscle Function	Continued supplementation for 7d post-exercise
Alessio et al. (1997)	Males	1000mg per day for 1d or 1000mg per day for 14d	Running at 80% $\dot{V}O_2$ max (30min)	↓ plasma TBARS (acute and prolonged suppl.)	
Nieman et al. (1997)	Male and female	1000mg per day for 7d and 1000mg before exercise	Running at 75-80% $\dot{V}O_2$ max (150min)	• serum IL-6 • serum cortisol • leucocyte function	Carbohydrate drinks given throughout run
Vasankari et al. (1998)	Males	500 mg pre-exercise, 500 mg during, and 1000mg post-exercise	19 km road race	↓ plasma conjugated dienes 1.5h post-exercise	No difference in conjugated dienes immediately post-exercise

Table 2.6   Vitamin C supplementation in humans.
↓ This parameter reduced in comparison to the control group. • No difference between supplemented and control groups.

Study	Subjects	Daily Supplementation	Exercise	Outcome
Witt *et al.* (1992)	Males	800IU vitamin E, 1000mg vitamin C, and 10mg β-carotene for 31 days	Cycling at 65% $\dot{V}O_2$ max (90min) on 3 consecutive days	↓ marker of oxidative damage to nucleic acids (urinary 8-hydroxyguanosine)
Kanter *et al.* (1993)	Males	888 IU vitamin E, 1000mg vitamin C, and 30 mg β-carotene for 6 weeks	Running at 60% $\dot{V}O_2$ max (30 min) and 90% $\dot{V}O_2$ max (5 min)	↓ Pentane and TBARS at rest and after exercise
Rokitski *et al.* (1994)	Males	400IU vitamin E and 200mg vitamin C for 31d	Marathon	● plasma TBARS ↓ serum CK

Table 2.7   Mixed antioxidant supplementation in humans.
↓ This parameter reduced in comparison to the control group. ● No difference between supplemented and control groups.

2.10.4 Other antioxidant molecules

Many other antioxidant molecules have received considerable attention recently, although rarely in relation to exercise-induced muscle damage. Carotenoids act as antioxidants both independently (Handelman 1996), and as pre-cursors for another antioxidant, vitamin A (Livrea *et al.* 1996). In a recent book, Karlsson (1997) devoted much of the text to the virtues of supplementation with ubiquinone (co-enzyme Q_{10}) in active individuals. Goldfarb (1999) recently suggested that flavenoids may prove to be very effective antioxidants in the future, including in respect to exercise-induced muscle damage. One final approach which warrants mentioning, is the administration of a combination of several antioxidants (Table 2.6).

2.11 Summary

The adverse effects of free-radicals are one of a number of potential causes of exercise-induced muscle damage and soreness. It appears that there is considerable scope for free-radical production both during and after exercise, and a number of investigators have suggested that radical-mediated damage may be an important factor. Therefore, the administration of appropriate antioxidants may diminish the extent of damage and soreness following demanding exercise. Furthermore, such an approach may also provide further insight into the extent of free-radical involvement in the damage process.

CHAPTER THREE

General Methods

3.1 Introduction

All experiments described in this thesis took place in the Exercise Physiology
Laboratories at Loughborough University, with each study being approved by the
University Ethical Committee. The specific design of each experiment is outlined in
subsequent chapters, whereas this chapter will deal with many of the methodologies
that were common between studies.

The subjects were male volunteers (aged 18-39), and were recruited by advertisement
within the University and the local area. Subjects were informed about the nature and
demands of each study, and were asked to give their written informed consent once they
fully understood the details of each study. Subsequently, subjects were required to
complete a medical history questionnaire (Appendix 1), and to provide information
about current weekly activity levels. Subjects with a medical condition that may have
interfered with the results of an experiment were excluded from the test. Smokers were
also excluded, since their vitamin C status may be different to non-smokers (Albanese
et al. 1975). Additionally, any subjects whose health may have been compromised by
taking part in a study were also excluded from participation in the test.

3.2 Anthropometry

Height was measured using a fixed stadiometer and recorded to the nearest 0.1 cm
(Holtain Ltd., UK). Subjects removed their shoes and stood with their heels together
and resting against the stadiometer. Subjects were asked to inhale deeply, and the
headboard lowered until it made contact with the superior point of the head. Gentle
traction was applied to the mastoid processes and occipital bone in order to compensate
for any shrinkage of the inter-vertebral discs.

Body mass was measured on a beam balance to the nearest 0.05 kg (Avery Ltd., U.K.). Subjects were required to wear only light clothing during this measurement, and to remain as stationary as possible. Before and after exercise, subjects were required to determine their own nude body mass, in order to calculate weight loss attributable to sweating. Upon completion of exercise, subjects were required to dry themselves prior to this measurement.

Skinfold thicknesses were measured to the nearest 0.1 mm using skinfold calipers (Holtain Ltd., UK). All measurements were made in triplicate on the dominant side of the body. Skinfold thickness was measured at four different sites: biceps, triceps, subscapular, and suprailiac. In order to determine the appropriate site for biceps measurement, subjects were requested to flex their arm to an angle of 90 °, and the midpoint between the acromion and olecranon processes was marked. The measurement of skinfold thickness was determined with the arm relaxed by the side of the body, with palms facing forwards. The measurement of triceps skinfolds was taken at the same level as the biceps, again with the arm relaxed by the side of the body. The subscapular was measured at the inferior angle of the scapular, also with arms in a relaxed position. Suprailiac skinfolds were measured superior to the iliac crest, with the arm slightly away from the body to facilitate easier access. Skinfold thicknesses are reported as the sum of the four sites.

3.3 Maximal oxygen uptake

The maximal oxygen uptake ($\dot{V}O_2$ max) of each subject was estimated using a progressive shuttle-run test (Ramsbottom et al. 1988). The test took place over a 20m distance, and the various levels of the test were indicated by an audio signal from a tape cassette. This cassette included a timed interval that ensured accurate playing speed. Subjects were motivated to continue until they could no longer sustain the required pace. The estimated $\dot{V}O_2$ max values were used to calculate the running speeds required to elicit 55% and 95% of $\dot{V}O_2$ max, according to the regression equation developed by Ramsbottom et al. (1988).

3.4 The Loughborough Intermittent Shuttle Test

The Loughborough Intermittent Shuttle Test (LIST) was designed to reflect the activity pattern commonly found in multiple-sprint sports (Nicholas *et al.* 1995; Nicholas *et al.* In Press). The overall protocol is depicted in Figure 3.1.
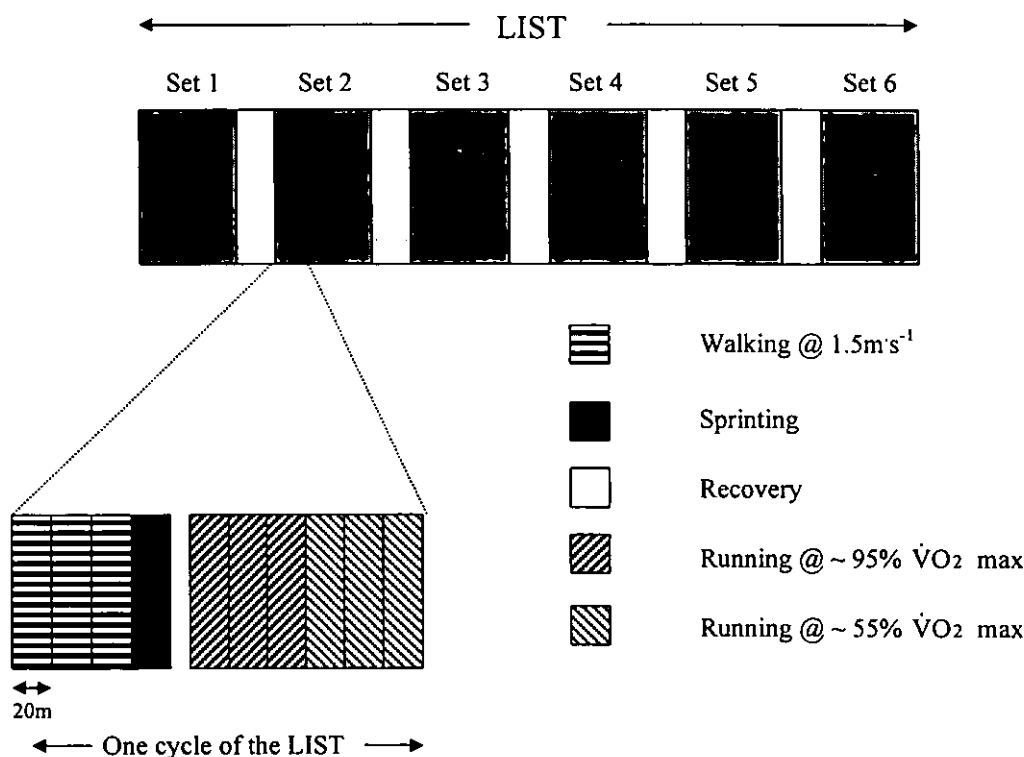
Figure 3.1   Schematic illustration of the Loughborough Intermittent Shuttle Test (LIST). Each set lasts 15 min, with a 3 min rest between sets. One cycle is repeated 11 times in each set.

The LIST was performed in the sports hall of Loughborough University, with the 20m distance required for the test clearly marked on the floor. The total exercise time of the LIST was always 90min, with a total rest time of 15 min. The different running and walking speeds were dictated by an audio signal from a computer (BBC Model B, UK), using software developed in this Department (Henryk K. A. Lakomy, Loughborough University). A different audio signal was also given to denote the mid-point of each

shuttle (10m), in order that pace could accurately be gauged. Maximal sprints were recorded over a 15m distance, which ensured that there was sufficient time to decelerate before having to change direction. Sprint times were recorded by two infra-red photo-electric cells (RS Components Ltd., UK), interfaced with the computer. Over the course of the LIST, subjects covered a total distance of 12.5km, sprinted approximately 1km, and changed direction 624 times. The approximate time spent performing the different activities is shown in Figure 3.2. The distances covered, and the proportion of time engaged in different activities during the LIST, compare very favourably with the available literature from match-analysis of football (Reilly and Thomas 1976; Mayhew and Wenger 1985; Bangsbo et al. 1991).
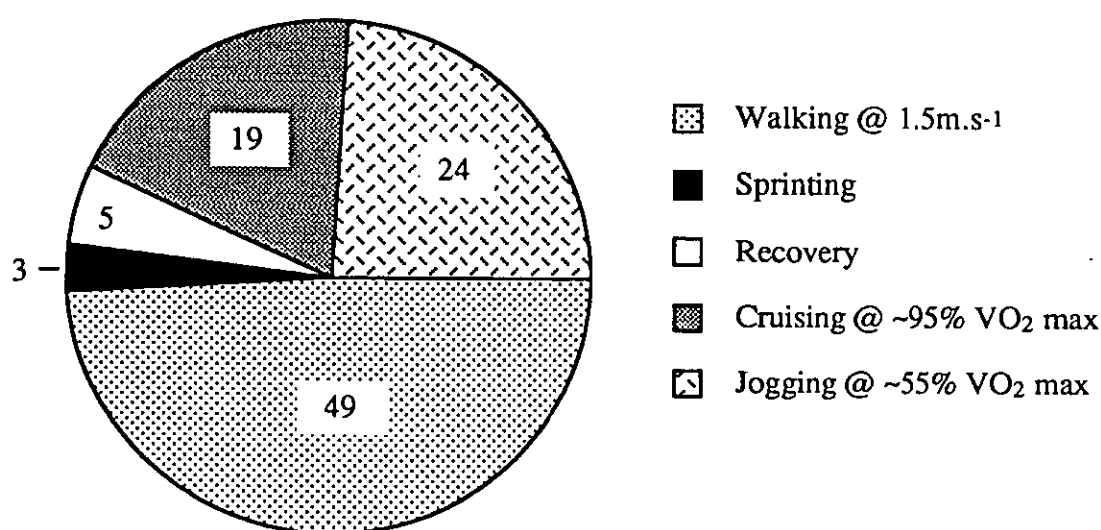
Figure 3.2   The approximate percentage of time spent working at each exercise intensity throughout the period of the LIST. Values do not include the 3 min rest between each set.

Heart rate was measured every 15s during exercise by short-range telemetry (Sports Tester, Polar Electro, Finland), and retrieved upon completion of exercise. During the last few minutes of each 15 min set, subjective ratings of perceived exertion were recorded using a 6-20 scale (Borg 1973). Subjects were allowed to consume water *ad libitum* throughout exercise, although the volume was monitored and used in the subsequent calculation of sweat loss. Ambient wet and dry bulb temperatures were

measured at the beginning and end of exercise using a whirling hygrometer (Brannan Thermometers Ltd., UK).

3.5 Soreness

Muscle soreness was assessed immediately before exercise, and then each morning over the following 3 days. Subjects were initially given a diagram of the body (Appendix 2) and a highlighter, and asked to label areas where they felt any soreness. Subjects were encouraged to palpate muscles to provoke the sensation of soreness. Subsequently, subjects were asked to rate the intensity of soreness in general, whole-body terms. In Chapter 4, soreness was assessed using an 11 point scale ranging from 0 = 'not sore' to 10 = 'very very sore'. In Chapters 5-7, the scale was reduced to a 10 point scale, from 1= 'not sore' to 10 = 'very very sore' (Appendix 3). This alteration was made since 10 point scales have been more been commonly used (Clarkson et al. 1987; Maughan et al. 1989; Gleeson et al. 1998a) than 11 point scales (Costill et al. 1990). In Chapters 6 and 7, a supplementary 100mm line was also introduced, which had no adjectives or divisions with the exception of 'normal' on the left, and 'very very sore' on the right (Appendix 3).

Intensity of soreness was also rated in individual muscle groups using either the 0-10 (Chapter 4) or 1-10 scales (Chapters 5-7) outlined above. Subjects performed one concentric action of each muscle group, and rated soreness in the active muscle (Tiidus and Ianuzzo 1983). The resistance during these contractions was set at approximately 75% of each subject's personal one-repetition maximum (1-RM), which was determined on a preliminary visit. Subjects were allowed three attempts at different weights in order to identify their true maximum. In Chapter 4, active soreness was assessed during a hamstring curl, squat, lateral pull, shoulder press and biceps curl (Unigym SSMG4 -7 and OPCM7, Powersport International Ltd., UK). In Chapters 5-7, active soreness was assessed during a hamstring curl, quadriceps extension, lateral pull, biceps curl and triceps extension (CYBEX models 4113, 4108, 5315, and 4005, LUMEX Inc., USA).

3.6 Muscle function

Concentric muscle function was assessed in the flexors and extensors of both legs on an isokinetic dynamometer (CYBEX model 770, LUMEX Inc., USA), interfaced to a computer operating CYBEX NORM software. On one of the preliminary visits to the laboratory, adjustments were made to ensure an ideal seating position for each subject. Every effort was made to ensure that movement was restricted to the saggital plane, and that the axis of rotation passed through the femoral condyles. Each subject's personalised set-up was stored on the computer and remained unchanged throughout the period of testing. All measurements were corrected for the effects of gravity. The dynamometer was calibrated (weight and speed) at the start of each study, according to the manufacturer's guidelines.

Subjects were familiarised with the muscle function test on at least two occasions during preliminary visits to the laboratory. Prior to all muscle function measurements, subjects were taken through a standardised warm-up consisting of 5 min gentle running, and 5 min stretching. Subsequently, subjects were seated and firmly secured on the dynamometer, in order to ensure that only the leg which was being tested contributed to the generation of force. There were five stages to the muscle function test: (1) ten sub-maximal contractions at a speed of 60 $°s^{-1}$, (2) three maximal contractions at 60 $°s^{-1}$, (3) two warm-up movements at a faster speed of 180 $°s^{-1}$, immediately followed by three maximal contractions at this speed (4) one five second isometric extension at an angle of 65 ° flexion, and finally, (5) one five second isometric flexion at an angle of 20 ° flexion. There was a 25 second rest period between each of these stages. The test always began with the same leg, although each treatment group contained equal numbers of subjects beginning with right or left legs.

3.6.1 Reliability of the muscle function test

The reproducibility of the muscle function test was assessed in a group of twenty male volunteers. Subjects were familiarised with the muscle function test on at least two occasions, before performing the test at similar times of day on two consecutive days. The mean age, height and body mass of these subjects were 24.3 ± 0.8 years, 178 ± 2

72

cm, and 82.1 ± 1.9 kg, respectively. The results of this investigation, including limits of agreement and correlation coefficients, are shown in Table 3.1. The presentation of the 95% limits of agreement is the preferred method for reporting differences between two measurements (Bland and Altman 1986). The mean difference between test one and test two (d), and the standard deviation of these differences (SD), was calculated. The 95% limits of agreement were calculated as d ± (1.96 x SD). There were no significant correlations between any of the differences (test one - test two), and the respective mean of these measurements. Therefore, heteroscedastic error was absent, and reporting the limits of agreement in absolute terms was appropriate (Nevill and Atkinson 1997).

Movement	Speed	Test 1 (Nm)	Test 2 (Nm)	Difference ± LOA (Nm)	Pearson correlation coefficient
Left Flexor	60° s^{-1}	140.2	140.8	- 0.6 ± 15.3	0.96
	180° s^{-1}	91.4	91.9	- 0.5 ± 15.7	0.94
	Isometric	149.1	151.5	- 2.4 ± 14.0	0.98
Right Flexor	60° s^{-1}	133.4	137.4	- 4.0 ± 15.5	0.96
	180° s^{-1}	92.6	95.0	- 2.4 ± 13.6	0.93
	Isometric	152.3	153.3	- 1.0 ± 14.8	0.97
Left Extensor	60° s^{-1}	213.0	212.3	0.7 ± 21.4	0.96
	180° s^{-1}	147.2	146.3	0.9 ± 18.8	0.90
	Isometric	268.1	267.0	1.1 ± 24.3	0.98
Right Extensor	60° s^{-1}	215.3	214.2	1.1 ± 21.6	0.96
	180° s^{-1}	143.3	145.5	- 2.2 ± 17.1	0.94
	Isometric	260.5	258.5	2.0 ± 24.2	0.98

Table 3.1   Test - re-test values for the muscle function test employed throughout this thesis. Values are means (n=20) based on peak torque (Nm). LOA: 95% Limits of Agreement

These results demonstrated that, in general, the muscle function test had a good level of reproducibility. There was some random variation (represented by the 95% limits of agreement), but little sign of a systematic effect or bias (represented by the mean difference between tests). The absolute levels of random variation appear to be similar, although the 95% limits of agreement expressed relative to absolute peak torque suggest that faster speeds are slightly less reliable. The correlation coefficients compare very well with previous studies that have investigated the reliability of isokinetic testing using similar speeds of movement (Rochcongar *et al.* 1988; Pincivero *et al.* 1997). Furthermore, the limits of agreement are similar to those reported for similar movements using different equipment (Nevill and Atkinson 1997).

3.7 Dietary restrictions

With the exception of the study reported in Chapter 4, subjects were always required to arrive in the laboratory after an overnight fast of at least 10h. In Chapter 4, subjects were asked to refrain from eating for at least 4h prior to reporting to the laboratory. On the day of exercise (Chapters 5-7), subjects were given a light meal exactly 2h before the LIST. This consisted of 1.7 g white bread and 0.3 g low-fat spread kg^{-1} body mass (Sainsbury's PLC., UK). Subjects also consumed 500 ml of an energy-free version of a commercially available drink, that contained 275 mg sodium (Lucozade-Sport, SmithKline Beecham, UK). The energy derived from this meal was 65% from carbohydrate, 23% from fat, and 12% from protein. This provided approximately 1.67 MJ, and 1g of carbohydrate per kg body mass.

In the studies reported in Chapters 5-7, subjects weighed and recorded their food and fluid intake for two days prior to exercise and for the three days after exercise. In Chapter 5, due to the cross-over design, subjects were asked to repeat this diet during their second trial. No restrictions were imposed regarding what could and could not be consumed during this period. In Chapters 6 and 7, subjects recorded their normal diet for a 5 day period at some point several days before the main testing period. During the two days before and three days after exercise in Chapter 6, subjects were prescribed

food to provide a diet very similar to normal, but including 100 mg vitamin C per day. In the study reported in Chapter 7, this was deemed to be inappropriate due to the extended period of supplementation. Therefore, subjects were allowed to eat freely, but were given a list of vitamin C portions of commonly available foods (Appendix 4). Over the two week period of supplementation and for the three days after exercise, subjects were required to consume 4 portions of these foods on a daily basis. It was estimated that this would provide approximately 100mg vitamin C per day. Weighed food records were analysed by a registered dietician using the software COMP-EAT 4.0 (Nutrition systems, UK), which is based on food composition tables (Paul and Southgate 1992). It has been estimated that accurate assessment of vitamin C intake requires between 6 and 33 days (Basiotis *et al.* 1987; Nelson *et al.* 1989).

3.8 Blood sampling

On the day of exercise (Chapters 5-7), blood samples were taken from a forearm vein using an indwelling cannula (Venflon, 18G, BOC Ohmeda, Sweden), and at all other times by venepuncture. The cannula was inserted under local anaesthetic (1% lignocaine, Antigen Pharmaceuticals Ltd., Ireland), and was kept patent with non-heparinised saline solution (0.9% Sodium Chloride, Steripak Ltd., UK). Over the 3 days after exercise, subjects returned to the laboratory at approximately the same time of day ($\pm$ 1h). Blood samples were always drawn after subjects had been standing for 15 min, as changes in body posture affect plasma volume (Rowell 1993). In all studies, blood was drawn into syringes and dispensed into blood collection tubes (Sarstedt Ltd., UK). Serum was collected by allowing whole blood to clot for 1 h (Chapter 4) or 20 min (Chapters 5-7), followed by chilled centrifugation (4 °C) at 3000 g for 15 min (Koolspin, Burkard Scientific Ltd., UK). The remaining whole-blood was added to tubes containing either lithium-heparin (Chapters 4 and 5) or ethylenediaminetetra-acetic acid (EDTA) (Chapters 6 and 7) as anticoagulants. Several small aliquots of blood (20 µl) were removed for the determination of lactate, glucose, haemoglobin and haematocrit (Section 3.9). The tubes containing anticoagulated blood were subsequently centrifuged at 3000 g for 15 min (4 °C) to obtain plasma, most of which

was dispensed and immediately frozen in liquid nitrogen. An aliquot of plasma (0.6 ml) was added to 0.6 ml of 10% metaphosphoric acid (Sigma Chemical Co. Ltd., UK), mixed, and immediately frozen in liquid nitrogen and stored at -70 °C until the supernatant was analysed for vitamin C.

In Chapters 5-7, approximately 3 ml of whole blood was dispensed into a tube containing EDTA, and transferred to tubes containing separation medium (Accuspin tubes, System Histopaque-1077, Sigma Chemical Co. Ltd., UK). These tubes were subsequently centrifuged at 1000 g for 10 min (at room temperature) in order to extract lymphocytes. Cells were washed once in 10 ml of phosphate-buffered saline (150 mmol.l^{-1} sodium chloride and 150 mmol.l^{-1} sodium phosphate, pH 7.2 at 25 °C) by centrifugation at 250 g for 10 min. The cell pellet was then re-suspended in 5 ml of phosphate-buffered saline (PBS), centrifuged, and centrifuged and washed a further two times before being finally re-suspended in 1 ml of PBS. A portion of this suspension (0.6 ml) was added to 0.6 ml of 10% metaphosphoric acid (Sigma Chemical Co. Ltd., UK), mixed, and immediately frozen and stored at -70 °C for vitamin C analysis. The remaining 0.4 ml was frozen and stored at -70 °C for subsequent determination of cell protein content, in order that the vitamin C content of lymphocytes could be expressed per g cell protein. The process of cell separation was always completed within 1 h (from the time blood was collected until the time of freezing).

3.9 Analysis of blood samples

Most biochemical analyses were performed in the Department of Physical Education, Sports Science and Recreation Management at Loughborough University. However, measurements of vitamin C and vitamin E (Chapter 5), and malondialdehyde (Chapters 5-7), were carried out in the Department of Medicine, Liverpool University. Additionally, interleukin-1β and interleukin-6 were measured by members of the School of Sport and Exercise Sciences, Birmingham University (Chapter 6), and interleukin-6 and C-reactive protein by Unilever Research, Colworth Laboratory

(Chapter 7). The details of all the methods which are not commercially available are given in the appendices.

Haemoglobin concentrations were measured by the cyanomethaemoglobin method (Boehringer Mannheim GmbH Diagnostica, Germany). Haematocrit was determined using a micro-haematocrit reader (Hawksley Ltd., UK), following centrifugation (15 min). Haemoglobin and haematocrit values were used to estimate changes in plasma volume (Dill and Costill 1974).

Glucose and lactate concentrations were determined from whole blood (20 µl) deproteinised in 2.5% perchloric acid (200 µl). Samples were mixed, centrifuged at 5000 g for 3 min (Eppendorf 5415C, Germany), and subsequently stored at -20 °C prior to analysis. Glucose concentrations were determined using a commercially available spectrophotometric method (Boehringer Mannheim GmbH Diagnostica, Germany). Lactate concentrations were determined using a modified fluorimetric method based on that described by Maughan (1982) (Appendix 5).

In Chapters 4 and 5, serum creatine kinase and aspartate aminotransferase activities were determined at 37 °C using commercially available spectrophotometric methods (Boehringer Mannheim GmbH Diagnostica, Germany). Similar spectrophotometric methods were used in Chapter 5, for the determination of serum uric acid and total iron concentrations (Boehringer Mannheim GmbH Diagnostica, Germany). These analyses, and the haemoglobin and glucose methods described earlier, were carried out on a standard spectrophotometer (Model CE393, Cecil Instruments Ltd., UK). In Chapters 6 and 7, much of the analysis was performed on an automated system (COBAS Mira Plus, Roche Diagnostic Systems, Switzerland) using similar methods. Serum creatine kinase and uric acid were determined using procedures developed for use with this system (Unimate, Roche Products Ltd., UK). In Chapters 6 and 7, serum myoglobin concentrations were determined using an immunoturbidimetric assay specifically developed for the automated system (Unimate, Roche Products Ltd., UK).

Serum cortisol concentrations were determined using a commercially available radioimmunoassay (Coat-A-Count, Diagnostic Products Corporation, USA). Radioactivity was measured using an automated gamma counter (Cobra II, Packard Instrument Co. Inc., USA).

The protein concentration of lymphocyte suspensions was determined using a bicinchoninic acid assay (Sigma Chemical Co., UK). Samples were allowed to defrost thoroughly before a 1% lauryl sulphate solution (Sigma Chemical Co., UK) was added (1:10), in order to fully solubilise proteins. Subsequently, 20 µl of sample was transferred into a microplate well. Standards were prepared by diluting a protein solution (1mg ml^{-1}, Sigma Chemical Co., UK) in phosphate buffered saline solution. Samples and standards were allowed to incubate overnight, before protein concentration was determined at 570 nm using a microplate reader (Benchmark, Bio-Rad, Japan).

3.9.1 High-performance liquid chromatography (HPLC)

The HPLC equipment in Chapter 5 (vitamin C and vitamin E), and in Chapters 5-7 (malondialdehyde), consisted of a pump (Model 303, Gilson, France), electrochemical detector (Model 141, Gilson, France), fluorescence detector (Model 121, Gilson, France), coupled to an integrator (Shimadzu C-R3A, Japan). The method for plasma vitamin E analysis was modified from that described previously (Woodall *et al.* 1996), using electrochemical detection rather than ultraviolet detection (Appendix 6). The method for malondialdehyde (MDA) analysis was based on that of Fukunaga *et al.* (1993), which is specific for MDA and avoids contamination from a number of different compounds (Appendix 7). Plasma and lymphocyte vitamin C analysis was performed using a method modified from Butcher (1991), using electrochemical detection rather than ultraviolet detection (Appendix 8).

In Chapters 6 and 7, vitamin C concentrations were determined using HPLC with ultraviolet detection and different procedures to those described above (Appendix 9). On this occasion, the HPLC equipment consisted of a pump (Model 302, Gilson,

France), ultraviolet detector (Pye Unicam Ltd., UK), coupled to an integrator (Model SP4290, Spectra Physics, USA). All vitamin C analyses until this point have referred solely to the ascorbic acid form. In Chapter 6, measurement of dehydroascorbic acid (DHAA) was also attempted by HPLC (Appendix 10). It appeared that DHAA was absent or in very low concentrations in the plasma of 4 subjects over the duration of the study, and therefore further analysis was not completed (Appendix 10).

3.9.2 Cytokines and C-reactive protein

In Chapter 6, plasma interleukin-1β (IL-1 β) and interleukin-6 (IL-6) were determined using solid-phase enzyme-linked immunosorbent assays (ELISA). The concentration of IL-1 β was measured using an ultra-sensitive commercially available method (Biosource International Inc., USA). The manufacturers claim that the minimum detectable concentration is 0.08 pg ml^{-1}, and the intra-assay coefficient of variation in the laboratory which performed this analysis was 4.6%. The concentration of IL-6 was determined using a similar commercially available method (Biosource International Inc., USA). The manufacturers claim that the minimum detectable concentration is 2 pg ml^{-1}, and the intra-assay coefficient of variation in the laboratory which performed this analysis was 6.0%. Plasma samples were stored with 20 μl of a protease inhibitor (Aprotinin, Sigma Chemical Co., UK).

In Chapter 7, serum was analysed for IL-6 using a commercially available solid-phase ELISA (Quantikine, R and D Systems Inc., UK). The manufacturers claim that this method is able to detect concentrations less than 0.7 pg ml^{-1}. The intra-assay coefficient of variation in the laboratory which performed this analysis was 4.2%. In Chapter 7, serum C-reactive protein was determined using a highly sensitive ELISA (Kordia Laboratory Supplies, Netherlands). The manufacturers claim that this method is able to detect concentrations less than 0.2 mg l^{-1}., and the intra-assay coefficient of variation in the laboratory which performed this analysis was 3.4%.

3.9.2 Coefficient of variation

The intra-assay coefficient of variation (SD/mean*100) for each assay is given in Table 3.2 (excluding cytokines and C-reactive protein). Each coefficient of variation was determined using at least 15 samples. The mean concentration for each series of analysis is also included in Table 3.2.

Assay	Units	Mean concentration	Number *	CV (%)
Lactate	mmol.l^{-1}	1.2	24	1.4
Glucose	mmol.l^{-1}	5.1	24	1.7
Uric acid (manual)	μmol.l^{-1}	344	15	2.8
Uric acid (automated)	μmol.l^{-1}	380	20	0.9
Total Iron	μmol.l^{-1}	23.6	15	1.9
Aspartate aminotransferase	U.l^{-1}	35	20	1.4
Creatine Kinase (manual)	U.l^{-1}	387	20	3.1
Creatine Kinase (automated)	U.l^{-1}	328	24	1.0
Myoglobin	μg.l^{-1}	109	15	5.4
Protein	g.l^{-1}	0.25	18	2.7
Vitamin C (chapter 5)	μmol.l^{-1}	51.4	15	5.7
Vitamin C (chapters 6-7)	μmol.l^{-1}	65.3	15	4.1
Vitamin E	μmol.l^{-1}	20.4	15	9.4
Malondialdehyde	μmol.l^{-1}	1.5	15	8.9
Cortisol	nmol.l^{-1}	314	15	6.5

Table 3.2   Intra-assay coefficient of variation.
* Number of replicate analyses on which intra-assay coefficient of variation was determined.

CHAPTER FOUR

MUSCULAR SORENESS FOLLOWING PROLONGED INTERMITTENT HIGH-INTENSITY SHUTTLE-

RUNNING

4.1 Introduction

Multiple-sprint sports are characterised by periods of high-intensity running, interspersed with lower intensity jogging or walking. Collectively, the number of people who participate in the multiple-sprint sports (e.g. soccer, rugby, and hockey), far outweigh those of other popular sports such as distance running (Williams 1990). Many individuals participate in these activities irregularly, with little or no long term preparation (Matheson 1991; Allied Dunbar National Fitness Survey 1992). Investigation of the physiological responses to multiple-sprint sports has been hampered in the past by the lack of an adequate laboratory-based model. Recently, Nicholas *et al.* (1995) developed a shuttle-running test based on the demands of multiple-sprint sports. Muscle function has been shown to be impaired immediately after performing this test (Nicholas 1996), and subjects often report having severe muscular soreness in the few days following the test. Notably, from earlier observations, even well trained individuals report muscle soreness after performing the test for the first time.

Therefore, the purpose of the present study was to assess soreness and markers of muscle damage after the unaccustomed performance of a prolonged, intermittent, shuttle-running test, designed to simulate the activity pattern commonly found in multiple-sprint sports.

4.2 Subjects and methods

Sixteen male students volunteered to take part in this study, and were randomly allocated to either an exercise or control group (Table 4.1). All participants were habitually active in a variety of sports, although none were soccer players, and none were familiar with the protocol used in the present study.

	Exercise Group	Control Group
Age (years)	23.8 ± 1.7	27.9 ± 1.6
Height (cm)	178 ± 2	179 ± 1
Body Mass (kg)	77.2 ± 3.2	80.9 ± 2.5
$\dot{V}O_2$ max (ml.kg^{-1}min^{-1})	55 ± 1	53 ± 2

Table 4.1:   Subject details in exercise and control groups.
                 Values are means $\pm$ SEM. (n=8 in each group).

Experimental design

The members of the exercise group performed the Loughborough Intermittent Shuttle Test (LIST) once (Chapter 3.4), while the control group rested. Heart rate was monitored continuously throughout exercise (Chapter 3.4). Subjects reported to the laboratory having refrained from strenuous physical activity for at least two days, and after a fast of at least 4 h. A 10 ml venous blood sample was taken from a forearm vein before and after either the control or exercise period, and then again approximately 24, 48, and 72 h later. Serum was analysed for creatine kinase and aspartate aminotransferase activities, and whole-blood for lactate concentrations (Chapter 3.9). Ratings of perceived soreness were measured before the test, and again approximately 24, 48, and 72 h later (Chapter 3.5).

Statistical analysis

Since serum muscle enzyme activity (CK and AST) was highly variable, a Friedman's two-way analysis of variance (ANOVA) was used to analyse control and exercise groups separately. A multiple-comparisons *post hoc* test (Seigel and Castellan 1988) was used to compare values within each condition. A one-way ANOVA with repeated measures was used to analyse parameters in the exercise group while performing the LIST. An independent two-way ANOVA with repeated measures was used to compare the remaining results between groups and over time. Where significant F ratios were found, a Tukey test was used to determine the cause of the variance. Parametric statistics were chosen to analyse the soreness results because of the lack of an appropriate non-parametric alternative. To ensure that the results of these tests were not influenced by the ordinal nature of the soreness data, we also performed a Friedman's two-way ANOVA to confirm the changes within each group. As the Friedman's ANOVA always agreed with the results of the two-way ANOVA over time, only the results of the parametric tests are referred to in the text. Relationships between variables were analysed using a Spearman's rank-order correlation. Significance was accepted at the 5% level. Values are expressed as means ± standard error of the mean (SEM), and medians (minimum-maximum).

4.3 Results

Heart rate during the test was only available for five subjects, and the mean for these individuals throughout the LIST was 172 ± 3 b. min^{-1} (n=5). Subjective rating of perceived exertion rose from 14 ± 1 at the end of set 1 to a value of 19 ± 1 at the end of set 6 (P<0.01). During the course of the LIST subjects drank 1.1 ± 0.1 l of water, and lost 1.7 ± 0.1 kg in body mass. Mean sprint times (15 m) were 2.49 ± 0.06 s in set 1, and were longer during sets 5 and 6 (2.56 ± 0.05 s and 2.58 ± 0.04 s, respectively) (P<0.05). Blood lactate concentrations were similar in exercise and control groups at rest (1.4 ± 0.3 mmol.l^{-1} *vs.* 1.2 ± 0.2 mmol.l^{-1}). After the LIST, however, lactate concentrations were significantly elevated in the exercise group compared with the control group (5.2 ± 0.5 *vs.* 1.1 ± 0.3 mmol.l^{-1}) (P<0.01).

Soreness

The perception of soreness before the test was minimal in both groups. Figures 4.1 and 4.2 show frequency of soreness for the exercise group only. The control group had similar pre-test soreness, with the hamstrings, quadriceps and gastrocnemius muscles each being rated as sore by one subject. For the 3 days after the LIST the control group had no soreness as measured by this method. The exercise group, however, showed a marked increase in soreness for the following 72 h. Soreness was not only confined to the weight-bearing muscles, but was also considerable in the upper-body. The hamstrings were the most affected part of the body, with all 8 subjects continuing to experience some soreness in this muscle group 72 h post-exercise (Figure 4.2).
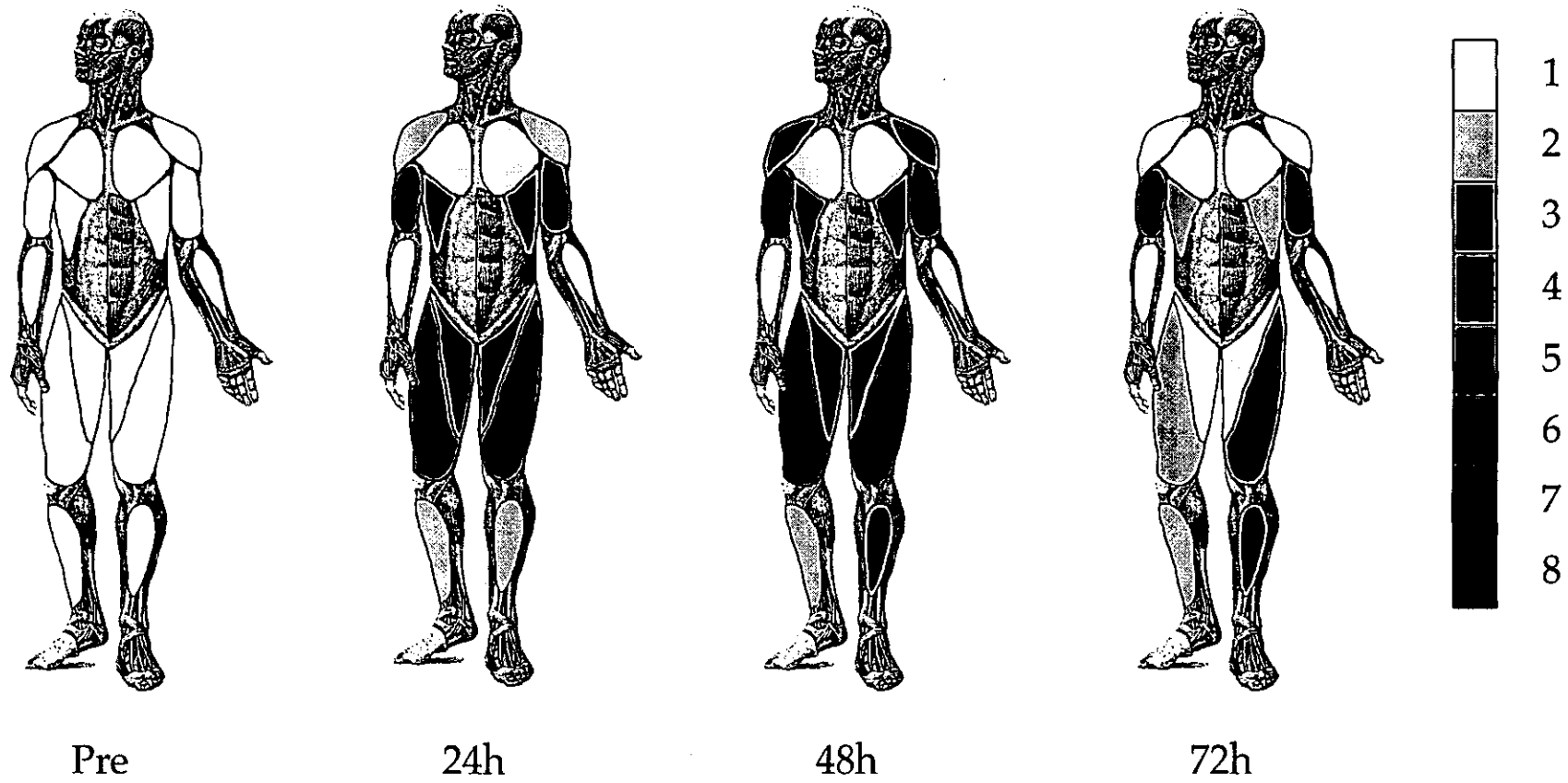
Figure 4.1   Anterior view of areas highlighted as sore before and after the LIST. An increase in the density of shading represents an increase in the frequency of soreness reported in that body part (n=8).
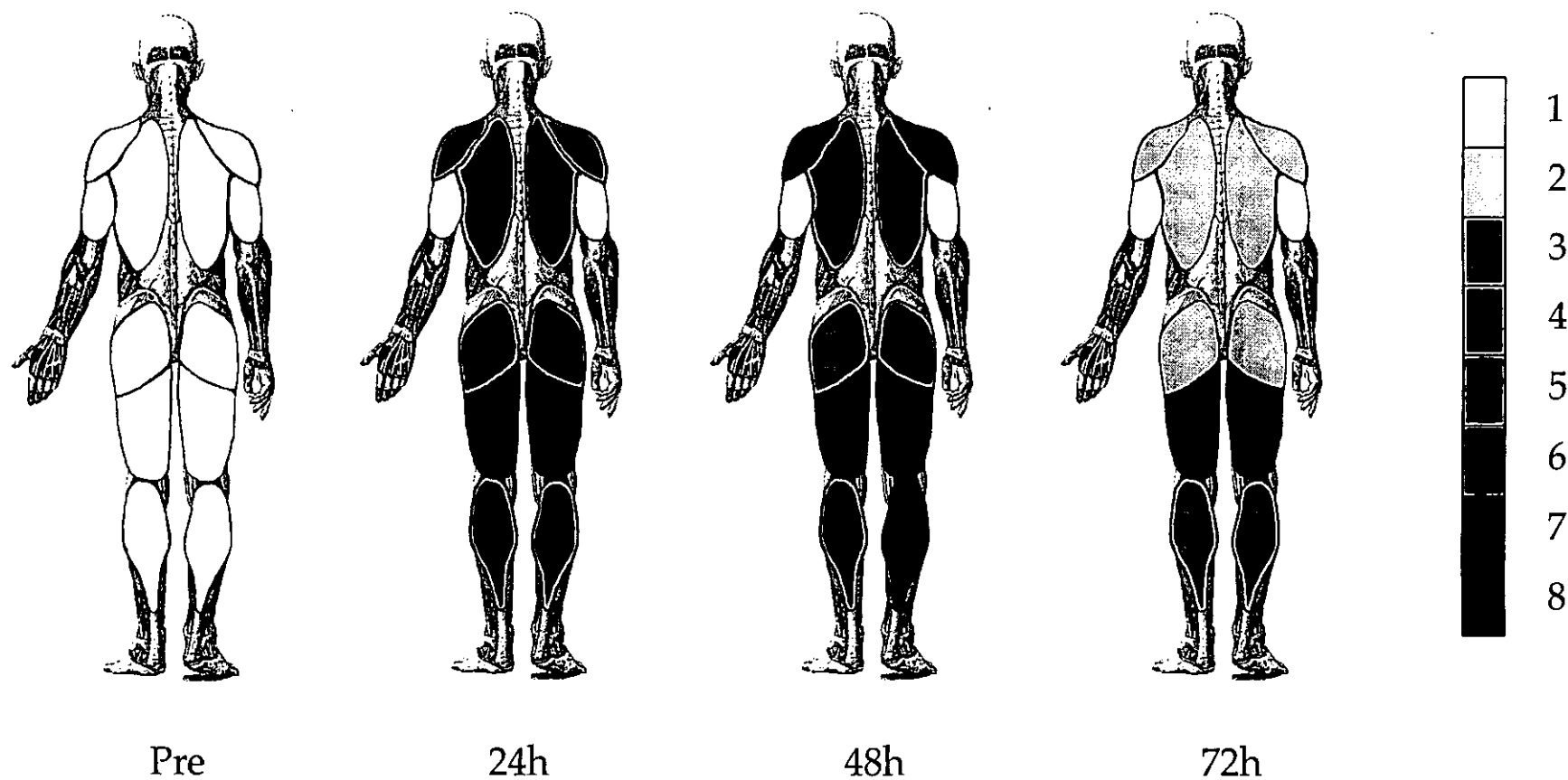
Figure 4.2   Posterior view of areas highlighted as sore before and after the LIST. An increase in the density of shading represents an increase in the frequency of soreness reported in that body part (n=8).

Intensity of both general soreness, and active soreness in the five parts of the body, were similar in exercise and control groups prior to the test (Figure 4.3 and Figure 4.4). In the 72 h after the test, both general and active soreness were higher than pre-exercise values in the exercise group, and were higher than all control values (P<0.05). In addition, soreness was rated higher at 24 and 48 h than at 72 h (P<0.05). Although the magnitude of soreness varied in the different parts of the body, the pattern of soreness was similar for all subjects. The hamstrings appear to be the most affected muscle group (Figure 4.3), reaching a recorded peak of 7 $\pm$ 1 units 48 h post-exercise. It is noteworthy that one subject could not lift the weights used for the determination of soreness in the hamstrings for the 3 days after exercise, due to weakness and pain in these muscles.
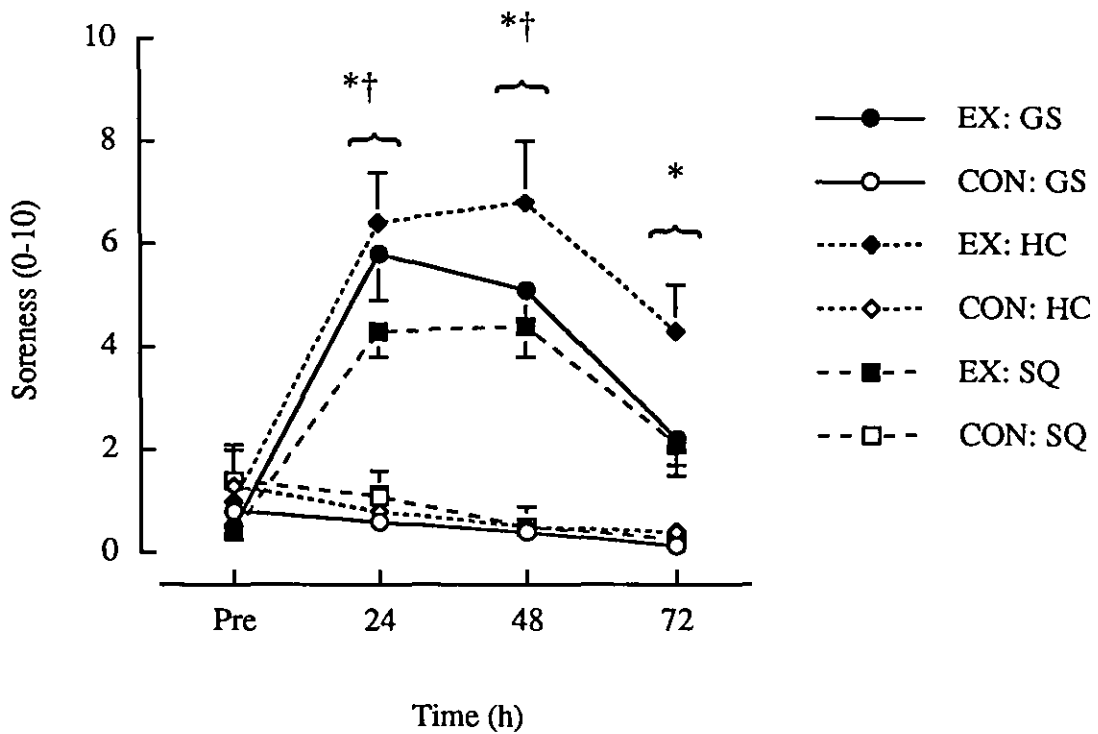
Figure 4.3   General soreness (GS), and soreness during a hamstring curl (HC), and squat (SQ), before and after the LIST or rest period. EX: exercise group (n=8), CON: control group (n=8). Values are means $\pm$ SEM. * All EX values greater than pre-exercise and all control values (P<0.05) † All EX values greater than 72 h post-exercise values (P<0.05).
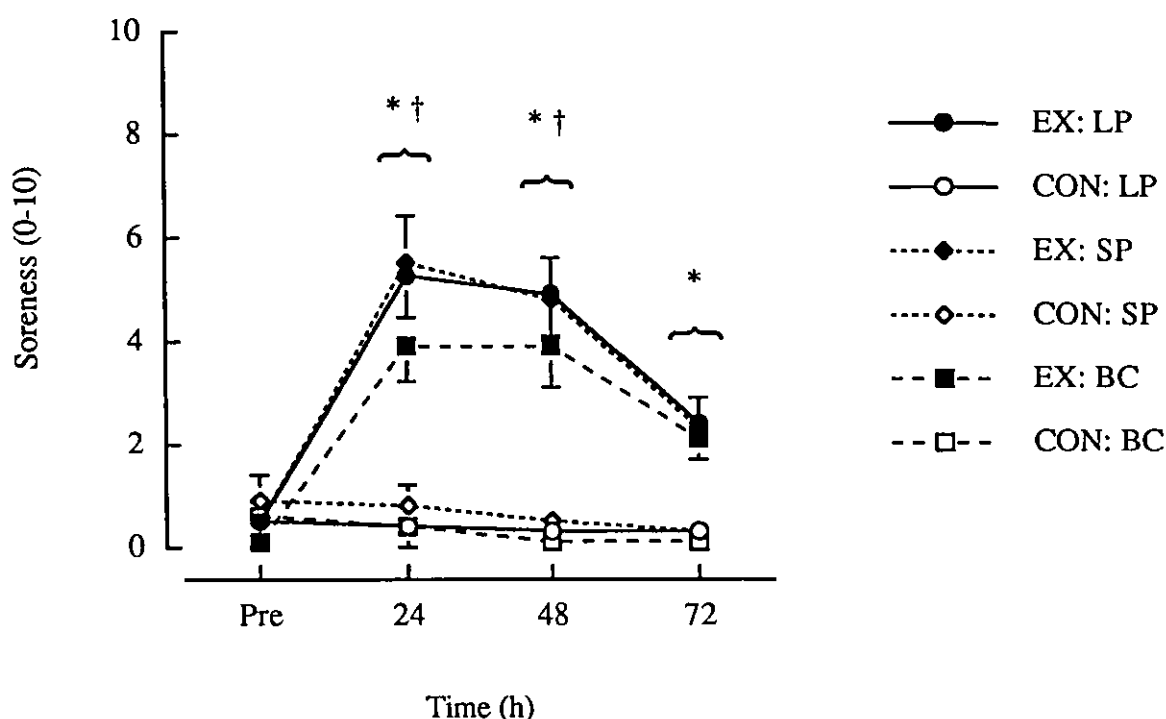
Figure 4.4   Soreness during a lateral pull (LP), shoulder press (SP) and biceps curl
(BC) before and after the LIST or rest period. EX: exercise group (n=8),
CON: control group (n=8). Values are means ± SEM. * All EX values
greater than pre-exercise and all control values (P<0.05) † All EX values
greater than 72 h post-exercise values (P<0.05).

Serum enzyme activity

Although eight subjects completed the LIST and eight others acted as controls, blood

samples were obtained from only seven individuals in each group. Baseline creatine

kinase (CK) and aspartate aminotransferase (AST) activities were similar in exercise

and control groups (Table 4.2), and did not change in the control group in the following

72h. In the exercise group, however, enzyme activities were elevated immediately after

exercise and remained so for the following 48h, peaking at 24h (P<0.01). The median

increase from baseline to 24h post-exercise was 379% for CK, and 93% for AST. All

subjects showed peak CK activity at this point, although there was considerable

variation in magnitude of response (range: 180-2109%). Six subjects showed peak AST

activity 24h after the LIST (range: 24-623%); the remaining subject showed peak

activity immediately post-exercise.

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Although there was a considerable increase in both soreness and the activity of muscle enzymes in serum, the relationship between these was not strong (Figure 4.5). There were poor correlation coefficients between general soreness and CK ($r= -0.36$), and AST ($r= -0.41$). Although there was considerable variation from individual to individual for both CK and AST activity, the two enzymes were related, and there was a strong correlation between them ($r= 0.95$) 24h after exercise ($P<0.05$). Neither peak soreness nor peak enzyme activity were related to age, body mass, maximum oxygen uptake, mean sprint time, or post-exercise lactate concentrations (Table 4.2).
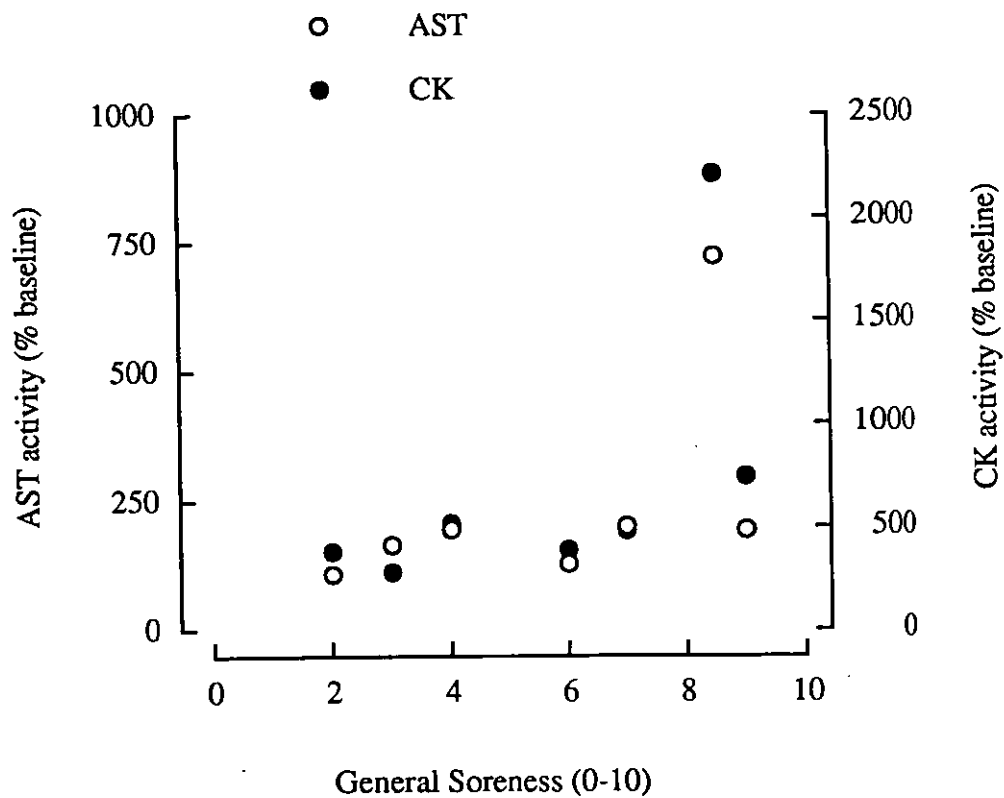
Figure 4.5   The relationship between aspartate aminotransferase (AST), creatine kinase (CK), and general soreness 24 h after exercise.

	Age	Body Mass	$\dot{V}O_2$ max	Mean Sprint Time	PE Lactate concentration
Peak Soreness	-0.09	-0.56	0.32	0.26	-0.61
Peak CK activity	-0.27	0.01	0.52	-0.33	-0.52
Peak AST activity	-0.40	-0.10	0.54	-0.29	-0.48

Table 4.2   Spearman's correlation coefficients between the parameters indicated for the exercise group only. PE: Post-exercise.

Time (h)		Pre-exercise	Post-exercise	24 h PE	48 h PE	72 h PE
CK	Control	127 (68-728)	130 (62-710)	92 (49-322)	103 (38-285)	105 (46-196)
(U.l^{-1})	Exercise	202 (78-521)	421 (240-1058) *	774 (580-5720) * †	391 (243-1908) *	254 (159-753)
AST	Control	23 (14-49)	22 (12-49)	22 (11-37)	21 (14-33)	24 (14-34)
(U.l^{-1})	Exercise	24 (15-41)	42 (23-52) *	43 (29-176) * †	36 (23-114) *	27 (15-68)

Table 4.3   Creatine kinase (CK) and aspartate aminotransferase (AST) activities in control (n=7) and exercise (n=7) groups
before and after the LIST or 1.5 h rest period. Values are medians (minimum - maximum). PE: Post-exercise.
* Greater than pre-exercise values (P<0.05) † Greater than 72 h post-exercise values (P<0.05)

4.4 Discussion

The Loughborough Intermittent Shuttle Test (LIST) requires participants to run and walk intermittently for 90 min, in an activity pattern representative of multiple-sprint sports. Unaccustomed performance of the LIST induced considerable soreness which lasted at least 72 h, but was greatest 24 to 48 h post-exercise. The activities of the muscle enzymes CK and AST in serum were highest 24 h after the test, although there was no correlation between either of these enzymes and the magnitude of soreness.

Our results are consistent with previous findings, which showed that soreness is typically highest 24 to 48 h after unaccustomed or strenuous exercise (Armstrong 1984; Ebbeling and Clarkson 1989). Soreness has been related to both the intensity and duration of the prior activity, with intensity playing the major role (Tiidus and Ianuzzo 1983). In the present study, there was considerable soreness in weight-bearing muscles and in the upper-body musculature. Soreness was greatest in the hamstrings, and persisted in all eight subjects for at least three days. Our results indicate, therefore, that a large proportion of the musculature is engaged during the LIST, with the hamstrings being particularly active.

All members of the exercise group showed a considerable increase in serum activity of both CK and AST the day following the LIST, the median percentage increase from baseline being 379% and 93%, respectively. The magnitudes of these increases were similar to those reported following exercise which produces muscle damage (Maughan et al. 1989; Rokitski et al. 1994a; Tsintzas et al. 1995). Downhill running, for example, increased mean serum CK and AST activities by approximately 380% and 110%, respectively (Maughan et al. 1989). The CK results are also similar to those found the day after a marathon, which may range from 496 $U.l^{-1}$ (Rokitski et al. 1994a) to 1146 $U.l^{-1}$ (Tsintzas et al. 1995). Rokitski et al. (1994a) also found AST activities following a marathon to be similar to the present study, being 42 $U.l^{-1}$ the day after exercise.

Some muscle enzymes may be released from all damaged muscles and, as a result, general whole-body soreness (GS) might be expected to be related to changes in CK and AST activity. Although muscle soreness and the activity of blood-borne muscle enzymes have been associated in the past (Schwane *et al.* 1983; Tiidus and Ianuzzo 1983), like others (Newham *et al.* 1986; Maughan *et al.* 1989), the present study failed to find a good relationship between the two. One explanation might be the extreme variability of markers such as CK, evidence for which is seen in the present and previous studies (Newham and Jones 1983; Maughan *et al.* 1989; Nosaka and Clarkson 1996). Enzymes such as CK and AST are found in extremely large quantities in muscle tissue. Creatine kinase is particularly abundant (approximately 2000 $U.g^{-1}$ muscle w.w.), with AST being much less so (approximately 40 $U.g^{-1}$ muscle w.w.) (Buyze *et al.* 1976). Damage to only 1 g of muscle may lead to significant changes in the levels of CK released into the systemic circulation (Volfinger *et al.* 1994). As a result, relatively small differences in the level of circulating enzymes between individuals may not reflect large physiological differences in the extent of muscle damage.

A recent study measured the release of skeletal troponin I (sTnI) following prolonged eccentric exercise, which by being almost entirely structurally bound, is not open to the same criticism as the use of cytosolic proteins (Sorichter *et al.* 1997). They followed the time course of sTnI and other markers of muscle damage, including CK, after both downhill running and prolonged eccentric contractions of the quadriceps. The results of this study showed that although CK release was not as rapid as sTnI, they generally increased in parallel. These findings suggest that CK is a good marker of muscle damage following different kinds of activity. Another recent study supports the use of enzymes such as CK in identifying whether muscle damage has occurred, as peak activity related strongly to damage as detected by magnetic resonance imaging (MRI) (Nosaka and Clarkson 1996). These authors found that following 24 maximal eccentric actions of the elbow flexors, a high serum CK activity was associated with a more profound MRI abnormality. It seems reasonable to conclude, therefore, that the changes in serum activity of CK and AST observed in the present study after the LIST reflect muscle damage.

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Although muscle soreness and muscle enzymes were elevated at the same time in the present study, these two parameters were not correlated. Muscle soreness and the appearance of muscle enzymes in the circulation may be the result of the same initial event, but follow an independent time-course. The efflux of muscle enzymes probably occurs as a direct result of the exercise bout, the delayed peak being partially explained by the time it takes for fibres to degenerate (Ebbeling and Clarkson 1989). Such a delay may also be the consequence of the slow transit through the lymphatic system, as creatine kinase does does not appear in the circulation until several hours after an intra-muscular injection of this enzyme (Volfinger et al. 1994). Delayed soreness, on the other hand, is reflective of conditions in the muscle at that particular time. Furthermore, comparison of any parameter with soreness is compounded by the subjective nature of pain. These factors, in addition to the large individual variability in the circulating levels of muscle enzymes, may explain the poor relationship between soreness and markers of muscle damage seen in the present study.

Soreness and muscle damage are typically more severe following eccentric, rather than concentric, work (Talag 1973; Newham et al. 1983). The LIST involves considerable acceleration and deceleration. Over the 90 min test, subjects performed 624 changes of direction, sprinted a total distance of approximately 1 km, and decelerated after the sprint for approximately 0.5 km. During these periods, the extent and magnitude of eccentric work was considerable. Friden et al. (1988) highlighted that running, especially sprinting, involves significant mechanical stress as muscles lengthen to decelerate the centre of mass. The incidence and magnitude of soreness was greatest in the hamstrings, which is probably related to the high demands placed on this muscle group throughout the LIST. The hamstrings work eccentrically to slow flexion of the hip, extension of the knee, and during the landing phase (Williams 1985). These factors probably explain why the present study found soreness and markers of muscle damage to be higher than seen previously after level running at a constant submaximal pace (Schwane et al. 1983).

Sports such as soccer, upon which the LIST is based, are both prolonged and of a high-intensity nature. This type of exercise is sufficient to impose considerable metabolic

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demands (Bangsbo 1994; Nicholas *et al.* 1995). Previous work in this laboratory has shown that following the LIST, muscle glycogen concentrations had fallen by 57% and 72% in type I and type II fibres, respectively (Nicholas 1996). Low levels of energy substrates have been proposed as a possible cause of muscle damage following a marathon (Warhol *et al.* 1985). Furthermore, exercise is associated with an increase in the production of possibly harmful free-radicals (Sjödin *et al.* 1990). At present, however, a metabolic cause of muscle damage and soreness following the LIST remains largely speculative.

In summary, there was a marked increase in both muscle soreness and markers of muscle damage following the LIST, whereas resting controls remained unchanged. Soreness was most intense 24 to 48 h post-exercise, although subjects remained sore for at least 72 h following the LIST. The activities of serum muscle enzymes were elevated for 48 h after exercise, showing the highest values the day after the LIST. It seems likely that, for individuals who partake in a multiple-sprint sport on an irregular basis (e.g. once a month), muscle soreness and damage are as common as the activity itself.

CHAPTER FIVE

MUSCLE SORENESS AND DAMAGE AFTER PROLONGED SHUTTLE-RUNNING FOLLOWING
ACUTE VITAMIN C SUPPLEMENTATION

5.1 Introduction

Unaccustomed performance of a shuttle-running test designed to simulate the activity
pattern of multiple-sprint sports led to considerable soreness and probable muscle
damage (Chapter 4). Muscle damage may be caused by a number of different factors
(Ebbeling and Clarkson 1989), including the adverse effects of free-radicals (Sjödin *et
al.* 1990; Kanter 1994; Jackson 1996).

During and after demanding exercise, endogenous antioxidant systems are
overwhelmed, since a number of studies have reported increased markers of free-radical
damage following several forms of activity (Maughan *et al.* 1989; Ashton *et al.* 1998;
Child *et al.* 1998c; McBride *et al.* 1998). Free-radical production may be increased
during exercise with a high metabolic cost (Kanter *et al.* 1993; Ashton *et al.* 1998), or
after the cessation of exercise during inflammation (Weiss 1989; MacIntyre *et al.* 1996;
Tiidus and Bombardier 1999). Furthermore, fluctuations in mitochondrial flux may
increase the proportion of oxygen that escapes normal tetravalent reduction, and
therefore contribute to radical formation (Del Maestro 1980). Consequently, there may
be considerable free-radical production during the Loughborough Intermittent Shuttle
Test (LIST), as it is metabolically challenging, of a variable intensity nature, and
apparently damaging (Chapter 4).

The provision of exogenous antioxidants has been shown to reduce muscle soreness
and damage after various modes of exercise (Jakeman and Maxwell 1993; Krotkiewski
et al. 1994; McBride *et al.* 1998). Vitamin C is one of the body's main antioxidants,
although very little experimental work has been carried out with this nutrient in relation
to exercise (Sen 1995; Goldfarb 1999). Ingestion of large amounts of vitamin C has

been shown to offer some protection against exercise-induced muscle soreness (Kaminski and Boal 1992), loss of muscle force (Jakeman and Maxwell 1993), and lipid peroxidation (Vasankari *et al.* 1998). Since vitamin C is water-soluble, availability may be increased after an acute single dose, and there may be no need for prolonged supplementation. This approach would offer a great deal of practicality, as supplementation may be undertaken in anticipation of demanding exercise and at short notice. Therefore, the aim of the present study was to determine whether soreness and muscle damage, experienced after prolonged shuttle-running, would be affected by vitamin C supplementation two hours before exercise.

5.2 Subjects and methods

Nine male students who neither smoked nor took vitamin supplements volunteered to take part in this study (Table 5.1). All participants were habitually active in a variety of sports, although none were familiar with the exercise protocol used in the present investigation.

Age (years)	Height (cm)	Weight (kg)	$\dot{V}O_2$ max $(ml.kg^{-1}.min^{-1})$
28.4 ± 1.3	175 ± 2	79.8 ± 4.0	55 ± 1

Table 5.1   Subject characteristics
                   Values represent the mean $\pm$ SEM (n=9).

Experimental design and procedures
The first bout of a novel exercise is more damaging than subsequent bouts of the same exercise (Byrnes *et al.* 1985). Therefore, at least 7 days prior to the first trial, subjects were familiarised with the Loughborough Intermittent Shuttle Test (LIST) for a 60 min period in order to minimise anticipated trial-order effects. The LIST protocol is described in detail in Chapter 3.4.

Subjects performed the LIST on two occasions, separated by exactly 14 days, although one subject performed the second trial 15 days after the first. In a double-blind and randomised fashion, subjects received either placebo or vitamin C (five subjects received vitamin C during their second trial). In the placebo trial, subjects were given a light standardised meal and drink exactly 2h before the start of exercise (Chapter 3.7). The drink comprised 500ml of an energy-free version of a commercially available drink (Lucozade-Sport, SmithKline Beecham, UK). During the vitamin C trial they received the same meal with 1g of ascorbic acid (Roche Products Ltd., UK) added to the drink immediately prior to consumption. The 2h time-delay between consumption of vitamin C and the beginning of exercise was chosen as a result of a pilot study (Appendix 11). Two hours was sufficient to increase serum vitamin C concentrations by approximately 100%, and values remained at this level for the time required to complete the LIST.

On the day of the test subjects arrived at the laboratory after an overnight fast of at least 10h. A resting blood sample was taken using an indwelling cannula (Chapter 3.8), after which subjects consumed the light meal (Chapter 3.7) and rested for 2h. Pre-exercise muscle function was assessed during this 2h rest period (Chapter 3.6). For 3 days after the LIST subjects returned to the laboratory after an overnight fast and at approximately the same time of day ($\pm$ 1h). A blood sample was taken from a forearm vein after the subject had been standing for at least 15 min. Subsequently, subjects rated the intensity of soreness (Chapter 3.5) and performed the muscle function test (Chapter 3.6). Food was weighed and recorded by the subjects for two days prior to the first main trial and three days afterwards, and they were instructed to eat the same diet over the course of the second trial. Subjects were also instructed to abstain from strenuous exercise for 2 days prior to performing the LIST, and not to resume exercising until the conclusion of testing. Serum was analysed for CK and AST activities, and also for uric acid, cortisol and total iron concentrations (Chapter 3.9). Plasma was analysed for vitamin C, vitamin E and malondialdehyde concentrations, and lymphocytes for vitamin C concentrations (Chapter 3.9). Additionally, whole-blood was used in the determination of haemoglobin and haematocrit values, and also lactate and glucose concentrations (Chapter 3.9).

Statistical analysis

A two-way analysis of variance with repeated measures was used to compare results between treatments and across time. Where significant F ratios were found, a Tukey test was used to determine the cause of the variance. When there were only single comparisons, a Student's t-test for correlated data was used to determine whether any differences between treatments existed. Variables were also analysed for trial-order effect irrespective of treatment, in order to ensure that the familiarisation had had the desired effect of minimising excessive responses after the first trial. Since there were no differences in terms of trial-order, only the results with regard to treatment are reported. Significance was accepted at the 5% level. Values are presented as means $\pm$ standard error of the mean (SEM). Due to difficulties in cannulating one subject, all the results from blood analysis are the mean of eight rather than nine subjects.

5.3 Results

Post-exercise blood lactate concentrations were similar in vitamin C and placebo trials, being 4.6 ± 0.5 and 4.7 ± 0.5 mmol.l^{-1}, respectively. Post-exercise blood glucose concentrations were 4.6 ± 0.2 mmol.l^{-1} in both trials. Mean heart rate and rating of perceived exertion were not different, being 169 ± 3 b. min^{-1} and 14 ± 1 in both trials. Environmental conditions were similar, ambient temperatures being 19.8 ± 1.2 and 20.1 ± 1.1 °C, and humidity 68 ± 3 and 69 ± 3%, during vitamin C and placebo trials, respectively. Total sweat loss was 2.1 ± 0.1 l after the vitamin C trial, and 2.3 ± 0.2 l after the placebo trial. There were also no differences in mean sprint times, which were 2.53 ± 0.04 and 2.55 ± 0.04 s in vitamin C and placebo trials, respectively. Sprint times in sets 5 and 6 were longer than in set 1 ($P<0.05$), irrespective of treatment. Plasma volume changes were not different at any point over the course of the experiment or between trials (Table 5.2). There were also no differences between trials in terms of dietary composition (Table 5.3).

	Baseline (- 2 h)	Pre	0.5 h	1 h	PE	24 h PE	48 h PE	72 h PE
VC	-------	4.5 ± 1.5	- 0.2 ± 0.9	- 0.4 ± 0.7	- 1.2 ± 1.2	4.4 ± 2.0	1.7 ± 1.7	4.1 ± 1.6
P	-------	3.5 ± 1.9	- 1.5 ± 2.0	- 0.9 ± 1.7	- 1.5 ± 1.7	3.0 ± 2.4	0.7 ± 1.5	2.7 ± 2.5

Table 5.2   Estimated percentage changes in plasma volume during vitamin C (VC) and placebo (P) trials compared with baseline. Values are means ± SEM (n = 9). Pre: pre-exercise, PE: post-exercise.

	Energy Intake (MJ)	CHO (%)	Fat (%)	Protein (%)	Alcohol (%)	Vitamin C (mg)	Vitamin E (mg)
VC	10.95 ± 0.67	52 ± 4	27 ± 2	16 ± 2	5 ± 3	120 ± 47	7 ± 1
P	11.16 ± 0.73	52 ± 3	27 ± 2	16 ± 2	5 ± 3	119 ± 52	8 ± 1

Table 5.3   Daily dietary composition during vitamin C (VC) and placebo (P) trials. Values are means ± SEM (n = 9). CHO: carbohydrate.

Plasma and lymphocyte vitamin C

Baseline resting plasma vitamin C concentrations were not different between trials (Figure 5.1), although 2 h after taking the supplement plasma levels were significantly higher in the vitamin C trial (P<0.01). Values continued to increase over the course of exercise in the supplemented trial, reaching a post-exercise peak of 199.8 ± 7.5 μmol.l^{-1}. Plasma vitamin C also increased to 88.9 ± 6.0 μmol.l^{-1} in the placebo trial after exercise (P<0.01). However, the day after the LIST, plasma vitamin C concentrations were similar to baseline values in both trials. Resting lymphocyte vitamin C concentrations were similar in both trials (Figure 5.1). However, immediate post-exercise lymphocyte vitamin C concentrations were above baseline and corresponding placebo values in the supplemented trial (P<0.05).
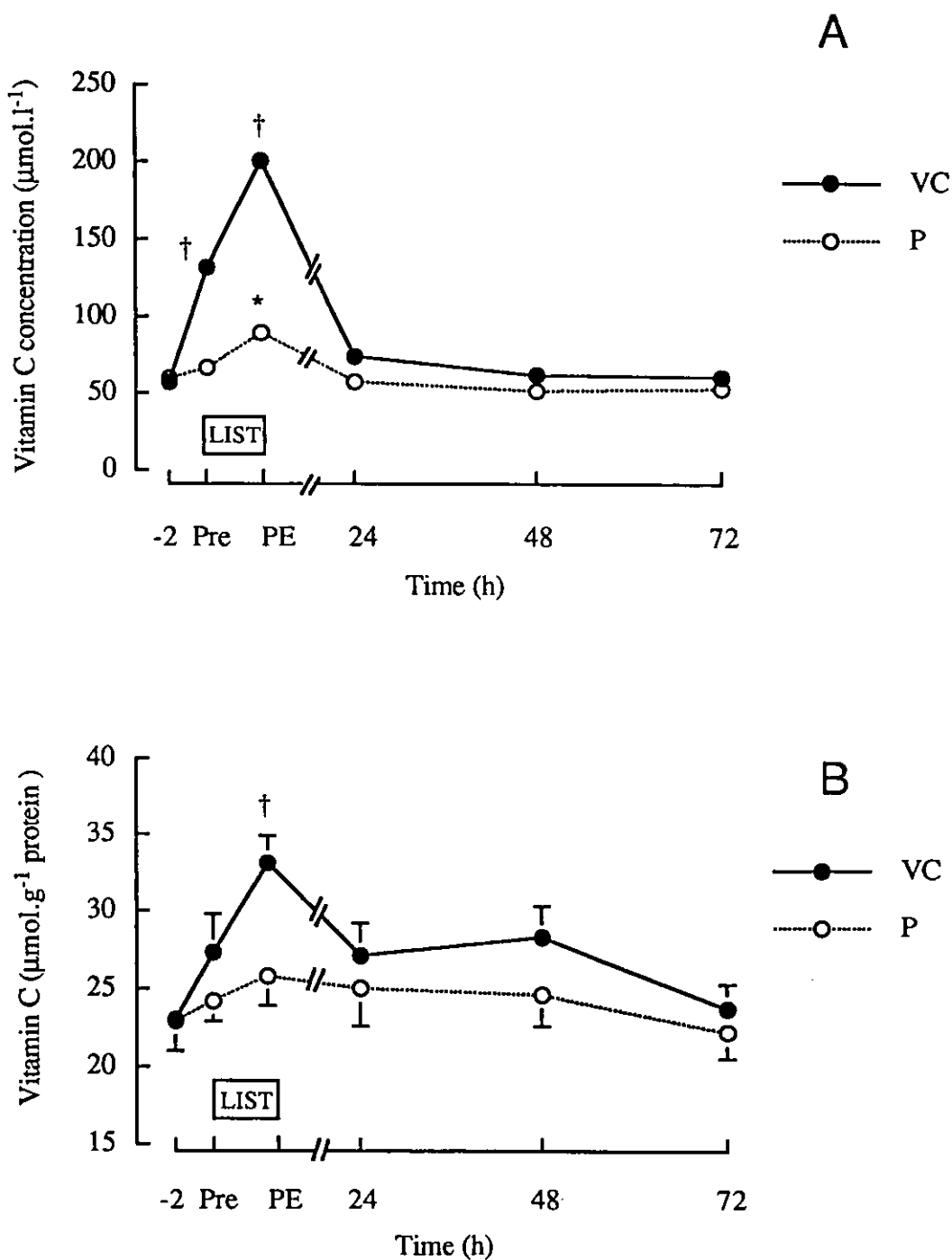
Figure 5.1   Vitamin C concentrations in plasma (A) and lymphocytes (B). Values
              represent means ± SEM (n=8). * Significantly above baseline values
              (P<0.01). † Significantly above placebo and baseline values (P<0.01).
              VC: vitamin C, P: placebo, -2: baseline, Pre: pre-exercise, PE: post-
              exercise, LIST: Loughborough Intermittent Shuttle Test.

Markers of muscle damage

Serum creatine kinase (CK) and aspartate aminotransferase (AST) activities are shown in Figure 5.2. The activity of both enzymes increased above baseline values during the 90 min of exercise ($P<0.05$). The activity of AST was highest immediately after exercise, whereas the activity of CK continued to increase in the post-exercise period and peaked 24h after the LIST. Peak AST represented an increase from baseline of approximately 75%, and peak CK an increase of approximately 270%. The activity of CK had returned to baseline 72 h after exercise, whereas AST remained elevated at this point. There were no differences between supplemented and placebo trials for either CK or AST at any time over the course of the experiment.

Plasma malondialdehyde

Plasma concentrations of malondialdehyde (MDA) increased after exercise in both trials by approximately 20% ($P<0.01$, Figure 5.3). There were no differences between trials for MDA, and no further differences over time.
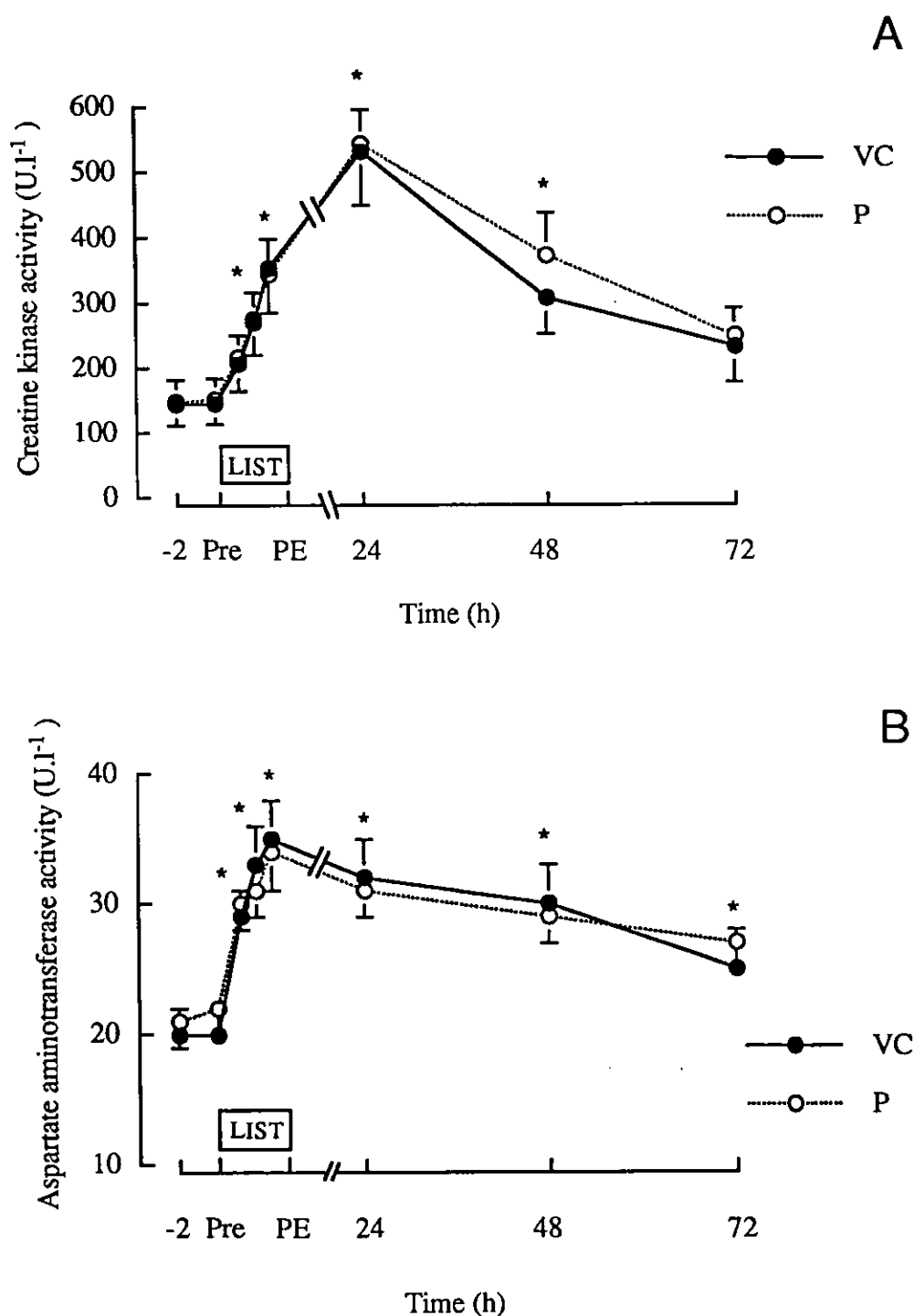
Figure 5.2   Serum activities of creatine kinase (A) and aspartate aminotransferase (B). Values represent means ± SEM (n=8). * Values in placebo and vitamin C trials above baseline values (P<0.01). VC: vitamin C, P: placebo, -2: baseline, Pre: pre-exercise, PE: post-exercise, LIST: Loughborough Intermittent Shuttle Test.

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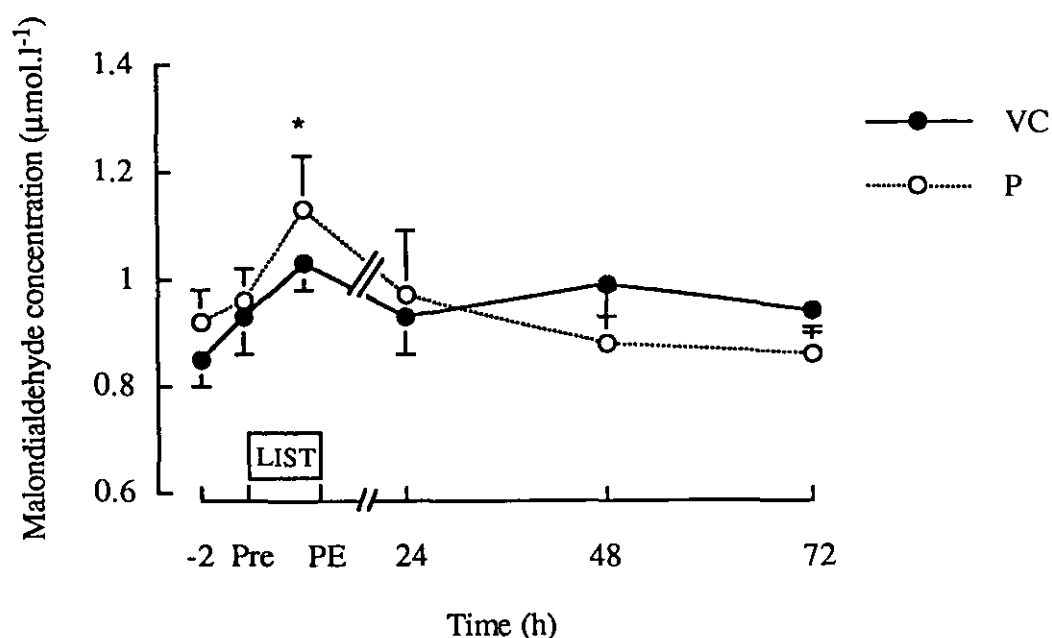
Figure 5.3   Plasma malondialdehyde concentrations. Values represent means ± SEM (n=8). * Values in placebo and vitamin C trials above baseline values (P<0.01). VC: vitamin C, P: placebo, -2: baseline, Pre: pre-exercise, PE: post-exercise, LIST: Loughborough Intermittent Shuttle Test.

Muscle function

Muscle function in the leg extensors did not change over the 3 days after the LIST, and was not different between trials (Table 5.4). However, leg flexor function was reduced in the placebo trial in the right leg, but only when performing an isometric contraction (P<0.05, Table 5.5). There was, however, a decline in muscle function in both left and right leg flexors during the vitamin C trial (P<0.05). This was observed up to 48h after exercise during both isometric and isokinetic contractions at the slower speed (60 °s[-1]).

		Left Extensor				Right Extensor			
		Pre - exercise	24 h PE	48 h PE	72 h PE	Pre - exercise	24 h PE	48 h PE	72 h PE
Isometric	VC	100	95 ± 4	99 ± 2	101 ± 3	100	96 ± 3	101 ± 3	100 ± 1
(%)	P	100	100 ± 3	100 ± 4	105 ± 3	100	98 ± 3	103 ± 3	104 ± 4
$60 \,^\circ s^{-1}$	VC	100	95 ± 3	96 ± 3	98 ± 2	100	95 ± 3	99 ± 3	101 ± 2
(%)	P	100	98 ± 2	99 ± 1	103 ± 2	100	99 ± 2	101 ± 2	103 ± 2
$180 \,^\circ s^{-1}$	VC	100	99 ± 3	104 ± 2	103 ± 2	100	97 ± 2	102 ± 3	100 ± 2
(%)	P	100	100 ± 2	100 ± 2	101 ± 3	100	101 ± 2	103 ± 2	104 ± 2

Table 5.4   Muscle function during vitamin C (VC) and placebo (P) trials in left and right extensors.
Peak torque is expressed as a percentage of pre-exercise levels. Values are means $\pm$ SEM (n = 9).
PE: Post-exercise.

		Left Flexor				Right Flexor			
		Pre - exercise	24 h PE	48 h PE	72 h PE	Pre - exercise	24 h PE	48 h PE	72 h PE
Isometric	VC	100	85 ± 4 *	89 ± 4 *	93 ± 5	100	87 ± 3 *	93 ± 4	97 ± 2
(%)	P	100	96 ± 5	96 ± 5	105 ± 3	100	95 ± 4 *	98 ± 4	102 ± 3
$60° s^{-1}$	VC	100	92 ± 3 *	92 ± 3 *	99 ± 5	100	94 ± 3 * †	98 ± 3	102 ± 3
(%)	P	100	99 ± 3	99 ± 2	105 ± 1	100	102 ± 3	100 ± 3	102 ± 2
$180° s^{-1}$	VC	100	94 ± 2	98 ± 2	97 ± 3	100	98 ± 2	100 ± 2	100 ± 3
(%)	P	100	100 ± 2	102 ± 3	105 ± 3	100	101 ± 3	98 ± 3	97 ± 4

Table 5.5   Muscle function during vitamin C (VC) and placebo (P) trials in left and right flexors.
Peak torque is expressed as a percentage of pre-exercise levels. Values are means ± SEM (n = 9).
* Below pre-exercise values (P<0.05), † Below placebo values (P<0.05).PE: post-exercise.

Muscle soreness

General soreness and leg soreness were both increased above baseline values 24 and 48 h after the LIST (P<0.01), although there were no differences between vitamin C and placebo trials (Table 5.6). There was little upper-body soreness in comparison to baseline values in either trial (data not shown).

		Pre - exercise	24 h PE	48 h PE	72 h PE
GS	VC	1 ± 0	4 ± 1 *	3 ± 1 *	2 ± 0
(1-10)	P	1 ± 0	3 ± 0 *	3 ± 1 *	2 ± 0
HCS	VC	2 ± 1	4 ± 1 *	4 ± 1 *	2 ± 1
(1-10)	P	2 ± 1	4 ± 1 *	3 ± 1 *	2 ± 1
LES	VC	1 ± 0	4 ± 1 *	2 ± 1	2 ± 0
(1-10)	P	2 ± 1	3 ± 1 *	3 ± 1	1 ± 0

Table 5.6   General soreness (GS) and soreness during a hamstring curl (HCS) and leg extension (LES) in vitamin C (VC) and placebo (P) trials. Values are means ± SEM (n = 9). * Above baseline values (P<0.01). PE: post-exercise.

Uric acid, cortisol, vitamin E and total iron

Serum concentrations of uric acid increased after the LIST (P<0.05), and were not different between trials (Table 5.7). Serum cortisol concentrations initially fell over the 2h after the light meal (P<0.05), and then increased over the course of exercise (Table 5.7). Immediate post-exercise cortisol values were above immediate pre-exercise values (P<0.05), and were not different between trials. Serum total iron concentrations increased by approximately 30% (P<0.05) over the 90 min of exercise in both trials (Table 5.7), although there were no differences between trials. Plasma concentrations of vitamin E did not change at any time in either trial (Table 5.7).

		Baseline (- 2 h)	Pre-exercise	0.5 h	1 h	Post-exercise	24 h PE	48 h PE	72 h PE
Uric Acid	VC	333 ± 14	317 ± 15	329 ± 11	338 ± 9	360 ± 11 *	327 ± 10	308 ± 14	312 ± 22
(μmol. l^{-1})	P	330 ± 17	329 ± 17	336 ± 17	361 ± 17	379 ± 17 *	342 ± 16	313 ± 16	325 ± 14
Cortisol	VC	501 ± 50	307 ± 29 *	463 ± 54 #	570 ± 39 #	683 ± 51 *#	493 ± 58	518 ± 46	506 ± 26
(nmol. l^{-1})	P	540 ± 50	332 ± 28 *	426 ± 41 #	543 ± 36 #	606 ± 38 *#	511 ± 52	492 ± 32	555 ± 60
Total Iron	VC	17 ± 2	19 ± 2	20 ± 2 *	23 ± 2 *	24 ± 2 *	19 ± 1	16 ± 1	15 ± 2
(μmol. l^{-1})	P	19 ± 2	21 ± 2	23 ± 3 *	24 ± 3 *	25 ± 3 *	19 ± 3	18 ± 2	16 ± 2
Vitamin E	VC	22.2 ± 2.8	19.0 ± 2.0	-	-	21.8 ± 2.3	19.3 ± 2.2	20.4 ± 1.6	22.8 ± 3.8
(μmol. l^{-1})	P	21.2 ± 2.9	17.0 ± 1.8	-	-	22.9 ± 4.0	19.9 ± 2.6	17.0 ± 1.5	20.2 ± 4.4

Table 5.7   Serum uric acid, cortisol, total iron and plasma vitamin E concentrations during vitamin C (VC) and placebo (P) trials. Values are means ± SEM (n=8). * Different to baseline values (P<0.05) # Above immediate pre-exercise values (P<0.01). PE: Post-exercise

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5.4 Discussion

The primary aim of this study was to investigate whether vitamin C, consumed two hours before exercise, would influence muscle soreness and damage following prolonged shuttle-running. Acute supplementation successfully increased plasma concentrations of vitamin C before and after exercise, and lymphocyte concentrations at the end of exercise. Nevertheless, supplementation with vitamin C had no effect on perceived muscle soreness, or markers of free-radical activity and muscle damage. Interestingly, the ability to generate force in the leg flexors the day after exercise was poorer following supplementation with vitamin C than after the placebo treatment.

Pre-exercise baseline plasma and lymphocyte vitamin C concentrations in the present study were similar to those reported previously (Gleeson *et al.* 1987; Robertson *et al.* 1991). Plasma vitamin C increased in the placebo trial at the end of exercise, as has been previously reported (Gleeson *et al.* 1987; Duthie *et al.* 1990; Rokitski *et al.* 1994a). Two hours after taking the supplement (pre-exercise), plasma concentrations of vitamin C were similar to those following several weeks of supplementation with daily 1 g doses of vitamin C (Witt *et al.* 1992; Kanter *et al.* 1993). Moreover, plasma vitamin C concentrations continued to increase over the exercise period in the supplemented trial, and reached a peak of approximately 200 μmol.l^{-1} at the end of exercise. This value is one of the highest reported in the literature, and is very close to values seen at a similar time point after an extremely high dose (5 g) of vitamin C taken at rest (Hornig *et al.* 1980). It is likely that plasma levels in the supplemented group after exercise represent the sum of both vitamin C delivered orally, and that released endogenously as a consequence of exercise. The high concentrations achieved may also reflect the fact that we administered the vitamin C with a light meal to prolong gastric emptying (Yung *et al.* 1981), which also contained sodium to facilitate the uptake of vitamin C (Karasov *et al.* 1991). Therefore, plasma concentrations of vitamin C respond rapidly to supplementation, and in this regard, prolonged supplementation may be unnecessary.

The post-exercise increase in markers of muscle damage (CK and AST) and soreness showed similar patterns to those reported previously for this type of activity (Chapter

4), although the increases were of a substantially smaller magnitude. This is probably due to the 1h familiarisation that preceded the first trial in the present study, as it is well known that a single bout of unfamiliar exercise protects against subsequent damage from the same type of exercise (Byrnes et al. 1985; Clarkson et al. 1987). However, the familiarisation succeeded in eliminating the trial-order effect we would have undoubtedly seen if it were not included. It is noteworthy that both soreness and markers of muscle damage increased substantially after exercise, and therefore the LIST led to muscle damage even after prior exposure to the test.

The efflux of muscle enzymes and the extent of muscle soreness, however, were not different between vitamin C and placebo trials. The similar increase in systemic levels of muscle proteins may indicate that vitamin C had no effect on sarcolemmal integrity, as differences in membrane permeability would lead to differences in circulating levels of CK and AST. This is supported, to some extent, by the similar increase in plasma malondialdehyde observed after both trials in the present investigation. Increased plasma concentrations of lipid peroxides after exercise may arise as a consequence of increased free-radical mediated damage to muscle cell membranes, although there are a number of other potential sources. However, increasing exercise intensity leads to greater levels of circulating lipid peroxides (Lovin et al. 1987; Kanter et al. 1993), which may imply that the working muscle was the major source of increased malondialdehyde in the present study. Unsaturated membrane fatty acids are particularly vulnerable to free-radical damage, and increased levels of sarcolemmal lipid peroxidation may lead to membrane permeability and the escape of muscle constituents (Sjödin et al. 1990; Jackson 1996). Elevated circulating concentrations of malondialdehyde have been observed after various modes of exercise, including running (Child et al. 1998c), cycling (Ashton et al. 1998), and resistance exercise (McBride et al. 1998). In the present study, therefore, prolonged shuttle-running increased free-radical production to the extent that antioxidant defences were overwhelmed, and lipid peroxidation occurred. Nevertheless, acute supplementation with vitamin C had no effect on the extent of lipid peroxidation.

There are several possible explanations for the lack of effect of vitamin C supplementation in the present study. One potential explanation is that the antioxidant defences of our subjects were adequate, and therefore increased availability of vitamin C offered no additional benefit. Active individuals appear to exhibit better vitamin C status than their less active counterparts (Fishbaine and Butterfield 1984; Robertson *et al*. 1991), and the subjects in the present investigation were all regularly active. Normal dietary vitamin C intake in the present study was approximately three times the reference nutrient intake, and was high enough to saturate most body compartments (Levine *et al*. 1996). Nevertheless, the increase in plasma malondialdehyde indicates that lipid peroxidation took place, and that endogenous antioxidant defences were insufficient to cope with exercise-induced free-radical production.

A more straightforward explanation may be that since the cause of damage to muscle during multiple-sprint sport type exercise has not been fully elucidated, free-radicals may not be involved. Theoretically, radicals are able to damage muscle tissue, cause an efflux of muscle enzymes, and decrease the ability to generate force, although so too are a number of other factors (Ebbeling and Clarkson 1989). It is possible that free-radicals were not the cause of the damage and soreness observed in the present investigation, and that another factor such as mechanical damage to muscle tissue was responsible (Ebbeling and Clarkson 1989). However, evidence from studies where antioxidants have reduced the extent of damage tend to support a role for free-radicals in the aetiology of muscle damage following exercise (Zerba *et al*. 1990b; Jakeman and Maxwell 1993; Krotkiewski *et al*. 1994; Rokitski *et al*. 1994a; McBride *et al*. 1998). Therefore, although it is unlikely that free-radicals are solely responsible for the muscle soreness and damage observed in the present study, oxidative stress is probably one of the key factors. However, the administration of vitamin C 2h prior to exercise appeared to be unable to moderate exercise-induced free-radical production.

Although plasma concentrations of vitamin C increased 2h after taking the supplement, lymphocyte concentrations were not elevated until after exercise. It has been suggested that leucocyte vitamin C concentrations reflect tissue levels (Jacob *et al*. 1987), and therefore cellular accumulation of vitamin C appeared to be delayed in the present

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study. This may reflect the need for ascorbic acid to be oxidised to dehydroascorbic acid before being transported across cell membranes (Vera *et al.* 1993; Washko *et al.* 1993; May *et al.* 1995). Since intracellular concentrations of vitamin C were not elevated until some time during exercise in the present study, much of the damage may have already been initiated. There is evidence that the damage process is well underway within the first few minutes of exercise (Lieber *et al.* 1996). Furthermore, neither plasma nor lymphocyte concentrations of vitamin C were above baseline the day after exercise, although it is unclear how rapidly concentrations of vitamin C returned to normal in the present study. It has been suggested that inflammatory processes are responsible for soreness (Smith 1991) and secondary muscle damage (MacIntyre *et al.* 1996). Therefore, it is possible that vitamin C concentrations had returned to normal at the time when provision of an exogenous antioxidant may have been of most benefit.

The cause of poorer muscle function in leg flexors following vitamin C supplementation is not entirely clear. The simplest explanation may be that these results are an example of a Type I error, although other explanations must also be considered. According to the Fenton reaction, ferrous iron may catalyse the production of the extremely reactive hydroxyl radical. Therefore, vitamin C potentially has pro-oxidant properties, since it is able to recycle the ferrous form of iron from the less reactive product of the Fenton reaction, ferric iron (Jenkins 1996). At the end of exercise there was a transient increase in serum iron concentrations, at a time when plasma vitamin C concentrations were also very high. However, in order for pro-oxidant reactions derived from vitamin C to be responsible for poorer muscle function in the present study, iron must have been available in the affected muscles. In support of this hypothesis, myoglobin is able to catalyse the Fenton reaction (Mitsos *et al.* 1988), and exercise also appears to increase the amount of loosely-bound iron in the muscles of rats (Jenkins *et al.* 1993). Therefore, high concentrations of vitamin C may have provoked pro-oxidant effects in the most active tissues in the present study. However, if this process had occurred, plasma indices of lipid peroxidation and muscle damage would also have been anticipated to be different. Potentially, this may reflect the failure of whole-body measurements to reflect localised differences, as vitamin C may have acted in a pro-oxidant manner in certain tissues, but as an antioxidant in others. Furthermore, there

may have been damage to intracellular components that was not reflected in markers such as CK, which are more closely associated with an increase in membrane permeability. Other investigators have shown that intracellular radical-mediated damage may be reflected by transient contractile dysfunction, which may occur in the absence of lipid peroxidation (Bolli *et al.* 1988; Kong *et al.* 1994).

Potentially, there are several other speculative explanations for poorer muscle function following vitamin C supplementation. Vitamin C may compete with glucose for entry into cells (Vera *et al.* 1993; Agus *et al.* 1997), and therefore high concentrations of vitamin C may hamper glucose uptake into muscle cells. Muscle damage may theoretically be increased as a consequence of decreased intracellular glucose concentrations, although only if this led to a pronounced fall in cellular levels of ATP (West-Jordan *et al.* 1990). However, it is unlikely that micromolar concentrations of vitamin C would interfere with the transport of millimolar concentrations of glucose. Furthermore, the GLUT 4 transporter responsible for glucose uptake into working muscle has an affinity for glucose 2-3 times that for vitamin C (Rumsey *et al.* 1997). An alternative explanation may be that because vitamin C is transported principally as dehydroascorbic acid, and reduced to ascorbate once inside cells (Agus *et al.* 1997), that a sudden intracellular increase in dehydroascorbic acid may compromise cellular reducing power. Therefore, considerable uptake of dehydroascorbic acid may have amplified demands on intracellular reducing agents (e.g. NADH or GSH), rather than contributing to them. However, such speculative explanations remain unsubstantiated, and the cause of poorer muscle function remains unclear.

In summary, acute supplementation with vitamin C two hours before exercise increased plasma concentrations of this vitamin before and after exercise, and lymphocyte concentrations at the end of exercise. However, muscle soreness, markers of muscle damage, and lipid peroxidation were elevated to an equal extent after exercise in placebo and supplemented trials. Therefore, although there was evidence of increased free-radical activity, which is believed to be a factor in damaging exercise, acute supplementation had no beneficial effects. Since cellular concentrations of vitamin C were not increased until after exercise, and the day after the test neither cellular nor

plasma concentrations were above baseline values, it is possible that supplementation occurred at an inappropriate time.

CHAPTER SIX

MUSCLE SORENESS AND DAMAGE AFTER PROLONGED SHUTTLE-RUNNING FOLLOWING POST-EXERCISE VITAMIN C SUPPLEMENTATION

6.1 Introduction

There is considerable muscle damage immediately after certain forms of exercise, which may be largely the product of mechanical factors (Ebbeling and Clarkson 1989; Armstrong *et al.* 1991). However, there have been a number of reports that the extent of muscle damage increases post-exercise, which is clearly unrelated to continued mechanical stress (Friden *et al.* 1983; Kuipers *et al.* 1983; Newham *et al.* 1983b; MacIntyre *et al.* 1996). It has been suggested that such secondary damage is related to an inflammatory response to the initial muscle damage (MacIntyre *et al.* 1996), which may also be the primary cause of delayed-onset muscle soreness (Smith 1991). A number of authors have proposed that destructive phagocytes may be responsible for secondary injury, since they are unable to distinguish healthy tissue from injured tissue (Weiss 1989; Smith 1994; MacIntyre *et al.* 1996). Activated neutrophils and macrophages release a number of destructive agents into the extracellular medium, including the superoxide radical (Weiss 1989). Additionally, xanthine oxidase may also contribute to post-exercise oxidative stress (Duarte *et al.* 1993; Radak *et al.* 1995), and also initiate inflammation (Granger 1988; Hellsten *et al.* 1997). Consequently, the efficacy of exogenous antioxidant supplementation may be the greatest after exercise, as this may be a time of considerable free-radical production and damage.

Plasma levels of vitamin C appear to fall after physical stress (Shukla 1969; Hume *et al.* 1972), including after demanding exercise (Gleeson *et al.* 1987). It is possible that vitamin C may be the most important antioxidant in regions of post-exercise free-radical production (Anderson and Lukey 1987; Frei *et al.* 1989; Chan 1993). Although previous studies have continued to supplement with vitamin C after damaging exercise (Kaminski and Boal 1992; Jakeman and Maxwell 1993), it is not clear whether

supplementation solely after exercise would provide the same physiological benefit. One thing that is clear, however, is that such an approach would have many practical advantages. Therefore, the aim of the present study was to investigate muscle damage and soreness following vitamin C supplementation after damaging exercise.

6.2 Subjects and methods

Sixteen male students volunteered to take part in this study, and were allocated to either a vitamin C or placebo group in a matched-group design (Table 6.1). Subjects who smoked or took vitamin supplements were excluded from the investigation. All participants were habitually active in a variety of sports, although none were familiar with the exercise protocol used in the present study.

	Age (years)	Height (cm)	Weight (kg)	$\dot{V}O_2$ max $(ml.kg^{-1}.min^{-1})$	Skinfolds (mm)	Weekly exercise sessions	Daily vitamin C intake (mg) *
VC	23.6 ± 1.4	179 ± 3	76.6 ± 4.6	55 ± 2	34.9 ± 5.0	3 ± 1	138 ± 30
P	24.3 ± 1.7	178 ± 3	79.3 ± 5.6	57 ± 1	35.9 ± 3.5	4 ± 1	151 ± 32

Table 6.1   Subject characteristics in vitamin C (VC) and placebo (P) groups. Values for each group represent the mean $\pm$ SEM (n=8). * Assessed prior to all testing.

Experimental Design and procedures

Subjects performed the Loughborough Intermittent Shuttle Test (LIST) on only one occasion (Chapter 3.4), to ensure that the exercise remained unaccustomed for all participants. On the day of the test subjects arrived at the laboratory after an overnight fast of at least 10h. A resting blood sample was taken using an indwelling cannula (Chapter 3.8), after which subjects consumed the light meal (Chapter 3.7) and rested for 2h. The muscle function test (Chapter 3.6) was performed before and immediately after exercise. For 3 days after the LIST subjects returned to the laboratory after an overnight fast, and at approximately the same time of day ($\pm$ 1h). A blood sample was taken from

a forearm vein after the subject had been standing for at least 15 min. Subsequently, subjects rated the intensity of soreness and performed the muscle function test (Chapter 3.5 and 3.6). Serum was analysed for CK and AST activities, and also for uric acid and cortisol concentrations (Chapter 3.9). Plasma was analysed for vitamin C, malondialdehyde, IL-1β and IL-6 concentrations, and lymphocytes for vitamin C concentrations (Chapter 3.9). Additionally, whole-blood was used in the determination of haemoglobin and haematocrit values, and also lactate and glucose concentrations (Chapter 3.9).

For the 2 days prior to the main trial and 3 days afterwards, subjects weighed and recorded a prescribed diet similar to their own. However, the amount of dietary vitamin C was controlled, in order to provide 100mg per day. In addition to the 100mg all subjects received in their diets, subjects in the vitamin C group consumed 200mg of vitamin C, twice a day, for the 3 days after exercise. This dosage is associated with complete bioavailability (Levine *et al.* 1996), and prolonged intake at this level has had beneficial outcomes in the past (Jakeman and Maxwell 1993). Vitamin C (ascorbic acid) was administered dissolved in 500 ml of an energy-free version of a commercially available drink (Lucozade-Sport, SmithKline Beecham, UK), with the same drink without vitamin C being given to subjects in the placebo group. Vitamin C was added to the drinks immediately prior to consumption. The first drink was given immediately after completion of the LIST, and the second drink in the evening of the same day. For the following 2 days, subjects consumed their drinks in the morning and evening of each day. Subjects were instructed to abstain from strenuous exercise for 2 days prior to exercise, and not to resume exercising until the conclusion of testing. Upon completion of the experiment, subjects were asked to consider whether they had an impression of which group they had been assigned (vitamin C or placebo), or if they had no perception of which group they had been allocated.

Statistical analysis

An independent two-way analysis of variance with repeated measures was used to compare results between treatments and over time. Where significant F ratios were found, a Tukey test was used to determine the cause of the variance. When there were

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only single comparisons, a Student's t-test for correlated data was used to determine whether any differences between treatments existed. Certain results were not normally distributed (CK, myoglobin and interleukin-1β), and therefore these values were log transformed prior to ANOVA. Log transformation always resulted in a normal distribution, and therefore these ANOVA results are reported. This statistical approach has been used in the past for similar parameters (Cannon *et al.* 1990; Smith 1995; Bruunsgaard *et al.* 1997; Ostrowski *et al.* 1998a). Significance was accepted at the 5% level, and values are presented as means ± standard error of the mean (SEM).

6.3 Results

The physiological responses to exercise were similar between groups. Heart rate throughout exercise was 169 ± 3 b. min^{-1} in the vitamin C group, and 172 ± 3 b. min^{-1} in the placebo group. Mean rating of perceived exertion throughout exercise was the same, being 15 ± 1 in both groups. Blood glucose concentrations at the end of exercise were 4.1 ± 0.3 mmol.l^{-1} in the vitamin C group, being no different to the 4.2 ± 0.2 mmol.l^{-1} observed in the placebo group. Blood lactate concentrations at the end of exercise were also comparable in both groups (5.0 ± 0.6 *vs.* 4.4 ± 0.7 mmol.l^{-1} for vitamin C and placebo groups). Mean ambient temperature was similar in vitamin C and placebo groups (18.3 ± 0.5 *vs.* 18.6 ± 0.6 °C), as was relative humidity (64 ± 4 *vs.* 64 ± 5 %). Subjects in the vitamin C group consumed 1.5 ± 0.2 l of water during exercise and lost 2.0 ± 0.1 kg in body mass (after correction for fluid intake), which was not different to the placebo group, which consumed 1.8 ± 0.2 l of water and lost 2.2 ± 0.2 kg in body mass. Mean sprint time over the course of the LIST was 2.54 ± 0.07 s in the vitamin C group, which was not different to the mean time of 2.52 ± 0.03 s in the placebo group. Sprint times in set 6 were longer than in set 1 in both groups (P<0.05).

Plasma volume changes were not different between vitamin C and placebo groups at any point over the period of testing (Table 6.2). However, plasma volume increased in both groups 48-72h after exercise (P<0.05).

	Baseline (-2h)	Pre-	PE	1h PE	2h PE	24h PE	48h PE	72h PE
VC	------	-1.3 ± 2.2	0.0 ± 2.4	0.6 ± 1.9	0.6 ± 2.0	4.8 ± 2.0	6.3 ± 2.0 *	5.2 ± 2.5 *
P	------	1.6 ± 1.4	0.9 ± 1.0	1.7 ± 1.4	1.3 ± 2.2	2.8 ± 2.8	6.8 ± 3.1 *	6.3 ± 3.0 *

Table 6.2   Estimated changes in plasma volume over the testing period in vitamin C (VC) and placebo (P) groups. Values in each group are means ± SEM (n=8). * Different to baseline values (P<0.05). Pre: pre-exercise, PE: post-exercise.

There were no differences between groups in terms of dietary composition over the 5 day period of the test (Table 6.3). Furthermore, subjects in both groups successfully adhered to the prescribed diet, and daily vitamin C intake was close to the target of 100mg.

	Energy Intake (MJ)	CHO (%)	Fat (%)	Protein (%)	Vitamin C (mg)	Vitamin E (mg)
VC	12.47 ± 0.60	57 ± 2	29 ± 2	13 ± 1	99 ± 2	7 ± 1
P	12.04 ± 0.79	53 ± 1	31 ± 2	15 ± 1	95 ± 4	7 ± 1

Table 6.3   Daily dietary composition assessed over the main test in vitamin C (VC) and placebo (P) groups. Values in each group are means ± SEM (n = 8). CHO: carbohydrate

Plasma and lymphocyte vitamin C

Plasma vitamin C concentrations increased similarly over the course of exercise in vitamin C and placebo groups (Figure 6.1)(P<0.01). This was to be expected, as vitamin C was not given to the supplemented group until after exercise. Values remained significantly elevated in the placebo group for a further hour after the

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completion of exercise (P<0.01). Plasma concentrations of vitamin C increased above those seen in the placebo group 1h after consuming vitamin C (1h post-exercise), and remained significantly above placebo values for the following 72h (P<0.01). The highest observed plasma vitamin C concentration in the supplemented group was 92.2 $\pm$ 7.0 μmol.l^{-1}, which was 1h after exercise. Vitamin C in the plasma appeared to be solely in the ascorbic acid form, and if dehydroascorbic acid was present, it was in very low concentrations (Appendix 10).

Lymphocyte vitamin C concentrations increased after exercise in both groups (Figure 6.1), and remained significantly above baseline levels until 24h after exercise (P<0.01). Although there was a tendency for lymphocyte concentrations to be higher in the vitamin C group after taking the supplement, values were not significantly different at any time.
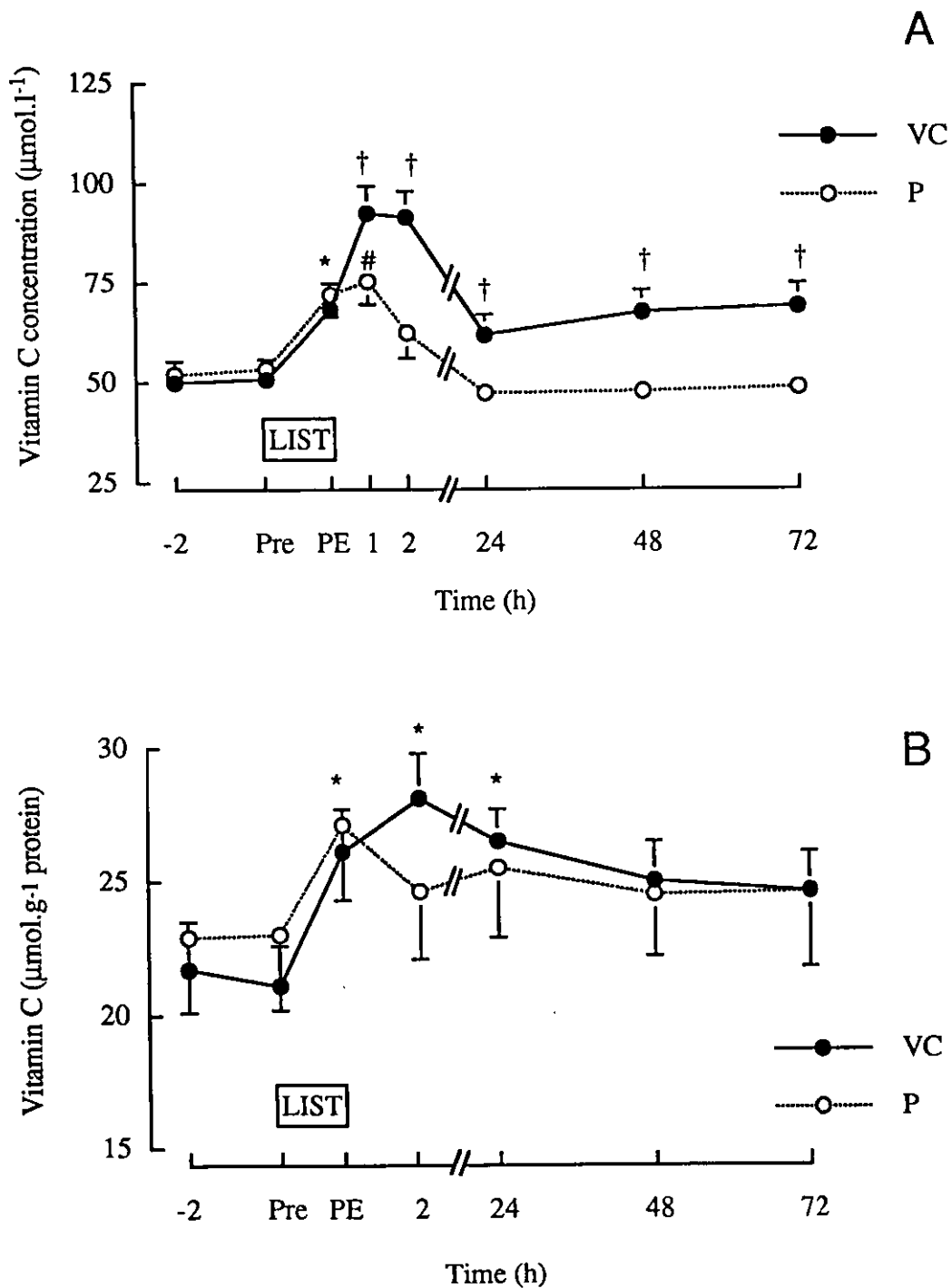
Figure 6.1   Plasma (A) and lymphocyte (B) vitamin C concentrations. Values
represent means ± SEM (n=8). * Placebo and vitamin C groups above
baseline values (P<0.01), † Vitamin C group above placebo values and
baseline values (P<0.01), # Placebo group above baseline values
(P<0.01). VC: vitamin C, P: placebo, -2: baseline, Pre: pre-exercise, PE:
post-exercise, LIST: Loughborough Intermittent Shuttle Test.

Markers of muscle damage

Serum activities of creatine kinase were elevated above baseline levels immediately after exercise in both groups (P<0.01), although peak activity was not seen until 24h after exercise (Figure 6.2). Creatine kinase remained significantly above pre-exercise values until 72h post-exercise, and were not different between groups at any point. Serum myoglobin concentrations also increased above pre-exercise values immediately after exercise (P<0.01), and appeared to peak 1h post-exercise (Figure 6.2). Myoglobin concentrations in both groups remained 60-90% above baseline values 24h after exercise (P<0.01), and every subject had elevated serum myoglobin concentrations at this point.

Plasma malondialdehyde

Plasma malondialdehyde concentrations were above baseline values in both groups at the end of exercise (Figure 6.3), and remained above baseline values until 24h after exercise (P<0.05). There were no differences between vitamin C and placebo groups at any time throughout the test.
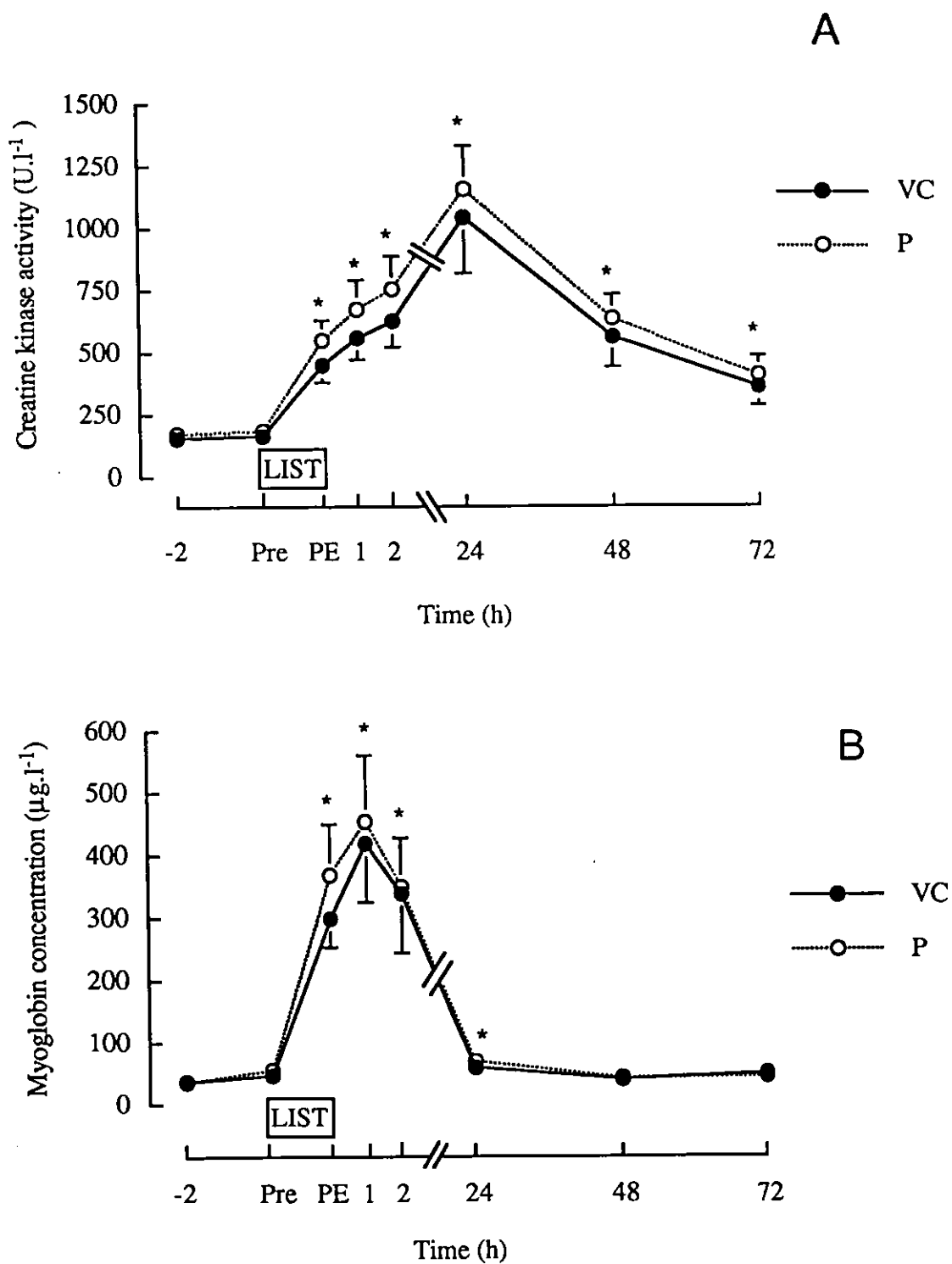
Figure 6.2   Serum creatine kinase activities (A) and myoglobin concentrations (B).
Values represent means ± SEM (n=8). * Placebo and vitamin C groups
above baseline values (P<0.01). VC: vitamin C, P: placebo, -2: baseline,
Pre: pre-exercise, PE: post-exercise, LIST: Loughborough Intermittent
Shuttle Test.
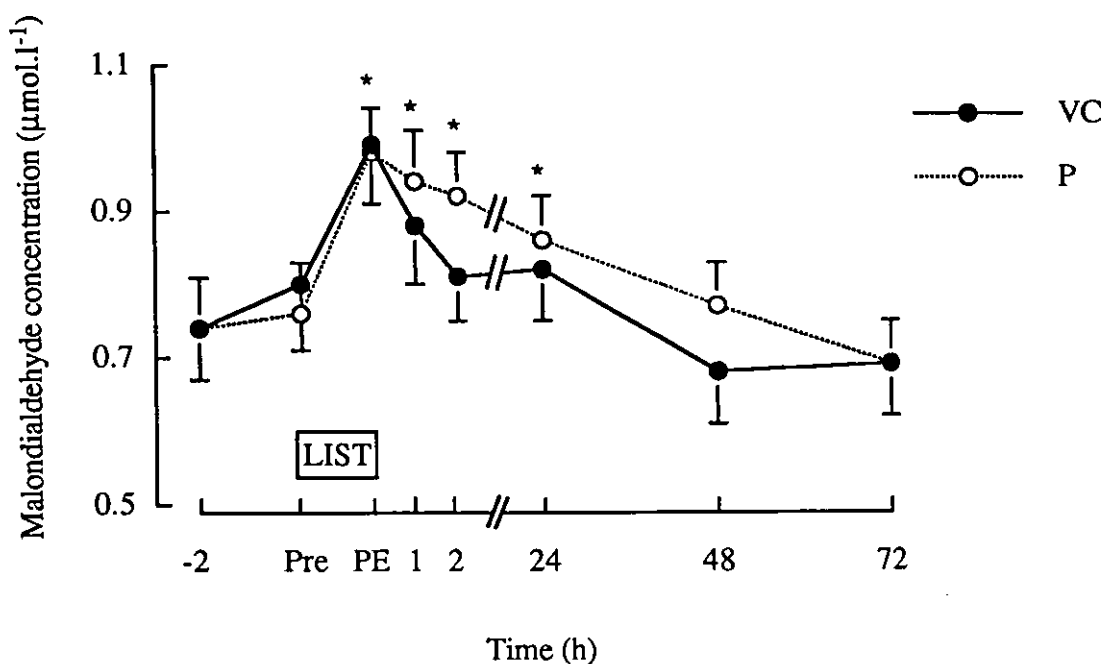
Figure 6.3   Plasma malondialdehyde concentrations (MDA). Values are means ±
              SEM (n=8). * Placebo and vitamin C groups above baseline values
              (P<0.05). VC: vitamin C, P: placebo, -2: baseline, Pre: pre-exercise, PE:
              post-exercise, LIST: Loughborough Intermittent Shuttle Test.

Muscle function

Muscle function was not significantly different between groups in either the leg flexors
or extensors at any time throughout the testing period (Tables 6.4 and 6.5). However,
right and left leg flexors exhibited reduced levels of muscle force in both groups after
the LIST (Table 6.4). Maximal isometric force was below baseline levels in both leg
flexors for 72h after exercise (P<0.01). This finding was similar at a speed of 60 $°s^{-1}$,
although the reduction in force was only significantly below baseline for 48h after
exercise (P<0.01). At the faster speed of 180 $°s^{-1}$, muscle function was only
significantly below baseline levels immediately after exercise (P<0.05).

The decline in muscle function in the leg extensors was also considerable (Table 6.5).
Maximal isometric force was below baseline levels immediately after exercise in both
groups (P<0.05). Isometric leg extensor torque remained impaired until 24h post-

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exercise in the right leg (P<0.05), and 48h post-exercise in the left leg (P<0.05). Maximal isokinetic muscle function at a speed of 60 $°s^{-1}$ was impaired until 48h after exercise in both left and right legs (P<0.05). At the faster speed of 180 $°s^{-1}$, values in both legs were only below baseline levels immediately after exercise (P<0.05).

		Left Flexor					Right Flexor				
		Pre-exercise	Post-exercise	24 h PE	48 h PE	72 h PE	Pre-exercise	Post-exercise	24 h PE	48 h PE	72 h PE
Isometric (%)	VC	100	83 ± 3 *	84 ± 2 *	85 ± 3 *	89 ± 4 *	100	85 ± 4 *	84 ± 4 *	89 ± 3 *	90 ± 6 *
	P	100	85 ± 4 *	84 ± 3 *	83 ± 3 *	88 ± 5 *	100	82 ± 5 *	78 ± 4 *	81 ± 3 *	88 ± 6 *
60 ° s^{-1} (%)	VC	100	91 ± 3 *	89 ± 4 *	91 ± 3 *	93 ± 3	100	85 ± 4 *	87 ± 3 *	94 ± 3 *	96 ± 4
	P	100	92 ± 3 *	92 ± 4 *	89 ± 6 *	96 ± 6	100	85 ± 3 *	87 ± 6 *	87 ± 4 *	91 ± 4
180 ° s^{-1} (%)	VC	100	90 ± 4 *	92 ± 5	95 ± 4	95 ± 4	100	90 ± 5 *	95 ± 2	92 ± 6	95 ± 8
	P	100	89 ± 7 *	94 ± 3	94 ± 3	95 ± 4	100	82 ± 5 *	88 ± 4	86 ± 4	88 ± 3

Table 6.4   Muscle function in vitamin C (VC) and placebo (P) groups in the left and right flexors. Peak torque is expressed as a percentage of pre-exercise levels. Values are means ± SEM (n = 8). * Below pre-exercise values (P<0.05).

		Left Extensor					Right Extensor				
		Pre - exercise	Post- exercise	24 h PE	48 h PE	72 h PE	Pre - exercise	Post- exercise	24 h PE	48 h PE	72 h PE
Isometric	VC	100	80 ± 4 *	88 ± 3 *	96 ± 6 *	97 ± 5	100	86 ± 2 *	90 ± 3 *	93 ± 4	96 ± 5
(%)	P	100	81 ± 4 *	88 ± 3 *	90 ± 3 *	93 ± 2	100	85 ± 3 *	93 ± 3 *	96 ± 4	96 ± 4
$60°s^{-1}$	VC	100	79 ± 5 *	88 ± 4 *	91 ± 4 *	94 ± 5	100	85 ± 3 *	89 ± 4 *	91 ± 4 *	93 ± 3
(%)	P	100	87 ± 3 *	94 ± 4 *	94 ± 3 *	94 ± 3	100	89 ± 3 *	94 ± 4 *	95 ± 2 *	95 ± 3
$180°s^{-1}$	VC	100	90 ± 3 *	96 ± 2	96 ± 2	99 ± 3	100	88 ± 1 *	94 ± 2	93 ± 2	96 ± 2
(%)	P	100	88 ± 5 *	94 ± 3	97 ± 2	97 ± 2	100	87 ± 5 *	97 ± 4	96 ± 3	95 ± 3

Table 6.5   Muscle function in vitamin C (VC) and placebo (P) groups in the left and right extensors. Peak torque is expressed as a percentage of pre-exercise levels. Values are means ± SEM (n = 8). * Below pre-exercise values (P<0.05).

Soreness

Muscle soreness increased in both vitamin C and placebo groups 24-72h after exercise (Tables 6.6 and 6.7). Interestingly, general soreness was higher at certain time points in the vitamin C group after the LIST (Table 6.6).

	Group	Pre-exercise	24h PE	48h PE	72h PE
GS (1-10)	VC P	1 ± 0 1 ± 0	6 ± 1 * † 4 ± 1 *	6 ± 1 * † 4 ± 1 *	3 ± 0 * 2 ± 0 *
GS (0-100)	VC P	1 ± 1 3 ± 1	55 ± 4 * 40 ± 9 *	59 ± 4 * † 29 ± 8 *	22 ± 4 * 17 ± 4 *

Table 6.6   General muscle soreness assessed on 1-10 (arbitrary units) and 0-100 (mm) scales. Values are means $\pm$ SEM (n=8). * Above pre-exercise values (P<0.01). † Above placebo values (P<0.05). VC: vitamin C, P: placebo.

There were 3 subjects in the placebo group who appeared to experience little whole-body soreness in relation to either the other members of the placebo group, or to the vitamin C group. The mean general soreness ratings for these individuals were 2 units (1-10 scale) and 13 mm (0-100mm scale) the day after exercise. Values for the remaining 5 members of the placebo group were very similar to those observed for the vitamin C group the day after exercise (6 units and 56 mm). The responses of these 3 subjects were lower than those seen in previous and subsequent experiments after unaccustomed performance of the LIST (Chapter 4 and Chapter 7), but did not appear to be related to the absence of muscle damage. The mean CK value for these subjects 24h after exercise was 1141 U.l^{-1}, and 1h after exercise their mean myoglobin concentration was 356 $\mu g.l^{-1}$.

	Group	Pre-exercise	24h PE	48h PE	72h PE
HCS	VC	1 ± 0	6 ± 1 *	6 ± 1 *	3 ± 1 *
(1-10)	P	2 ± 0	4 ± 1 *	4 ± 1 *	2 ± 0 *
LES	VC	2 ± 0	6 ± 1 * †	4 ± 1 *	3 ± 1
(1-10)	P	2 ± 0	3 ± 1 *	3 ± 1 *	2 ± 0
BCS	VC	1 ± 0	2 ± 0	2 ± 1	1 ± 0
(1-10)	P	1 ± 0	1 ± 0	1 ± 0	1 ± 0
TES	VC	1 ± 0	2 ± 0	2 ± 1	1 ± 0
(1-10)	P	1 ± 0	1 ± 0	2 ± 0	1 ± 0
LPS	VC	1 ± 0	3 ± 1 *	3 ± 1 *	1 ± 0
(1-10)	P	1 ± 0	2 ± 1 *	2 ± 1 *	1 ± 0

Table 6.7   Active muscle soreness in individual parts of the body, assessed on a 1-10 scale during each movement. HCS: hamstring curl soreness, LES: leg extension soreness, BCS: biceps curl soreness, TES: triceps extension soreness, LPS: lateral pull soreness. Values are means ± SEM (n=8). * Above pre-exercise values (P<0.01). † Above placebo values (P<0.01). VC: vitamin C, P: placebo.

Soreness in individual muscle groups also increased 24-48h after exercise (Table 6.7). The hamstrings and quadriceps were both greatly affected, and hamstring soreness persisted until 72h after exercise. Soreness did not significantly increase above pre-exercise levels in the biceps or triceps of either group. There was a tendency for soreness to be higher in the vitamin C group, although values were only significantly different 24h after exercise in the leg extensors (P<0.05). As was the case for general muscle soreness, this appeared to be largely due to 3 individuals in the placebo group with particularly low soreness ratings.

Cytokines

Plasma levels of interleukin-1β (IL-1β) were extremely variable at all times, and therefore individual values are shown in Figure 6.4. There were no differences between vitamin C and placebo groups throughout the testing period. There was a trend for values to be slightly higher after exercise in both groups, and IL-1β was significantly above baseline 1h after exercise (P<0.05). Thirteen subjects had higher levels of IL-1 β

at this time, although the extent of the increase varied considerably. Plasma levels of interleukin-6 were mostly below the detection limit of the assay used in the present study, including after exercise (Appendix 14).
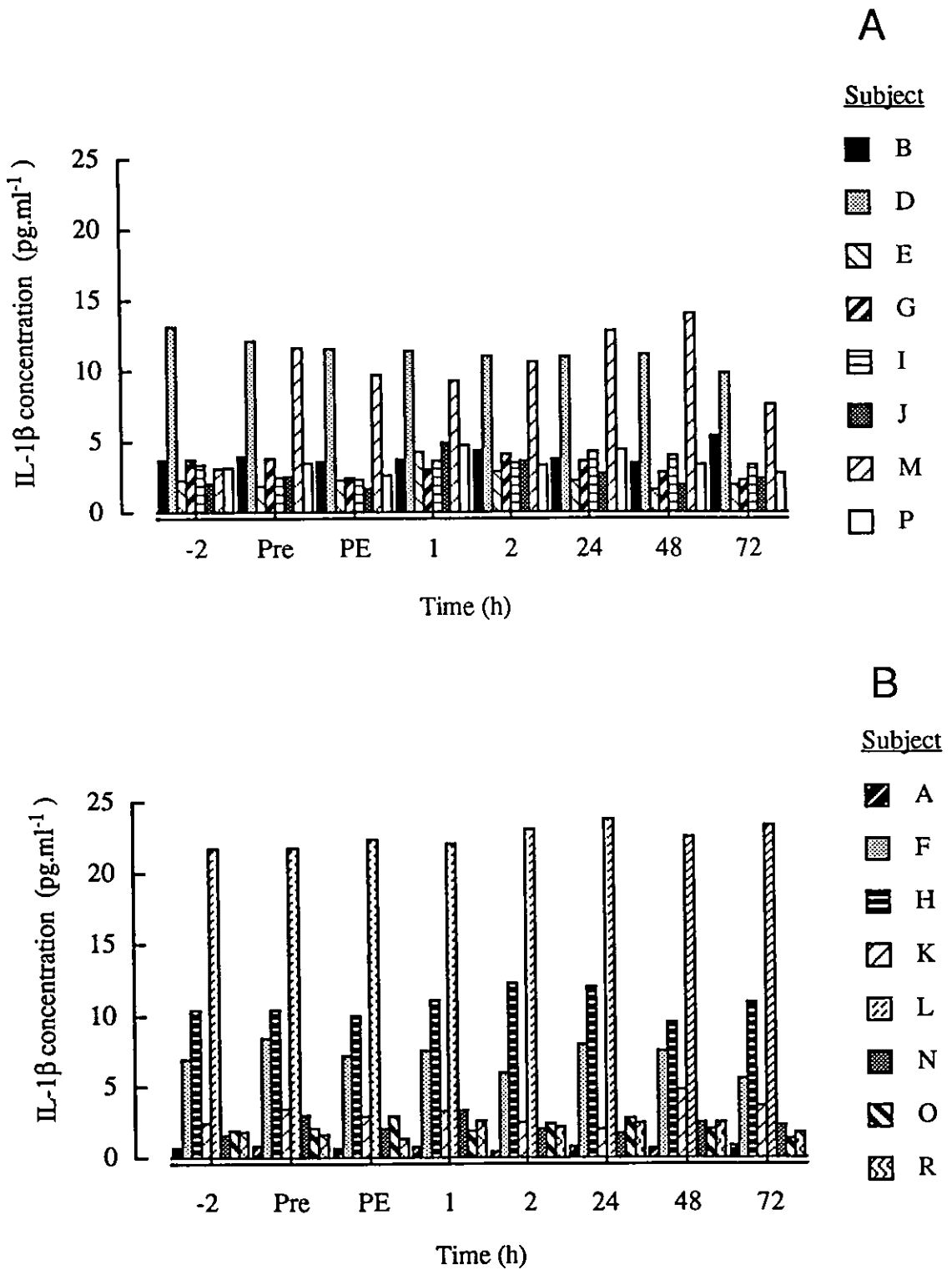
Figure 6.4   Plasma interleukin-1β concentrations for vitamin C (A) and placebo (B)
groups. Values are given for each individual. -2: baseline, Pre: pre-
exercise, PE: post-exercise.

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		Baseline (-2h)	Pre-exercise	Post-exercise	1h PE	2h PE	24h PE	48h PE	72h PE
Cortisol	VC	521 ± 186	334 ± 85 *	639 ± 154 **	529 ± 170 **	374 ± 159	474 ± 149	447 ± 175	467 ± 193
(nmol.l^{-1})	P	581 ± 83	299 ± 24 *	707 ± 198 **	608 ± 201 **	473 ± 206	523 168	517 ± 145	533 ± 164
Uric acid	VC	257 ± 16	277 ± 17	317 ± 18 *	318 ± 20 *	315 ± 25 *	285 ± 15 *	257 ± 11	230 ± 11
(μmol.l^{-1})	P	297 ± 20	314 ± 18	350 ± 22 *	363 ± 22 *	369 ± 24 *	334 ± 26 *	299 ± 22	289 ± 25

Table 6.8   Serum cortisol and uric acid concentrations before and after the LIST in vitamin C (VC) and placebo (P) groups. Values are means ± SEM (n=8) * Different to baseline values (P<0.01) ** Above immediate pre-exercise values. PE: Post-exercise, VC: vitamin C, P: placebo.

Cortisol and uric acid

Serum levels of cortisol were not different between vitamin C and placebo groups at any point (Table 6.8). Cortisol concentrations increased at the end of exercise in both groups, and remained above pre-exercise levels 1h after exercise ($P<0.01$). There were no differences in serum concentrations of uric acid between groups at any point over the testing period (Table 6.8). Uric acid concentrations increased above pre-exercise levels and remained elevated until 24h after exercise ($P<0.05$).

Perceived group

The perceived treatment group of all sixteen subjects is shown in Table 6.9. Subjects were unaware of which treatment they had received, although this did not prevent them forming an opinion about which group they had been assigned. Interestingly, none of the subjects in the vitamin C group perceived that they were in this group. Additionally, the 3 subjects in the placebo group who all reported soreness to be low considered themselves to be in the vitamin C group.

Subject perception of group	Vitamin C	Placebo
Perceived in VC group	0	4
Perceived in P group	5	2
No perception of group	3	2

Table 6.9   Perceived group (if any) at the end of the testing period.
VC: vitamin C group, P: placebo group.

6.4 Discussion

The aim of this study was to investigate whether vitamin C, consumed after demanding exercise, would influence the extent of soreness and damage that developed in the post-exercise period. Vitamin C supplementation had no effect on most parameters, including the circulating levels of muscle proteins, lipid peroxidation, and the loss of muscle force. An interesting finding, however, was that some aspects of soreness appeared to be higher in the supplemented group than in the placebo group.

Plasma concentrations of vitamin C increased at the end of exercise in the present investigation, which is in agreement with a number of previous studies (Gleeson *et al.* 1987; Duthie *et al.* 1990; Maxwell *et al.* 1993; Rokitski *et al.* 1994a). Supplementation increased plasma concentrations of vitamin C after this point, and therefore successfully increased the availability of this antioxidant within 1h of exercise. Consistent with the results of Dhariwal *et al.* (1991), there did not appear to be any vitamin C in the dehydroascorbic acid form (Appendix 10). Plasma concentrations of vitamin C have been reported to fall in the days following physical stress (Shukla 1969; Hume *et al.* 1972; Gleeson *et al.* 1987), although they did not change in the placebo group in the present study. It is likely that the provision of 100mg of vitamin C in the normal diet of the placebo group was sufficient to prevent any such decline from occurring.

Vitamin C was also determined in lymphocytes, which along with other leucocytes, are believed to reflect tissue concentrations of vitamin C (Jacob *et al.* 1987). Although there was a tendency for lymphocyte concentrations to be greater following supplementation, this difference was not statistically significant. This finding may be related to the relatively high vitamin C intake of our subjects, as they consumed 138 mg of vitamin C a day in their normal diet prior to supplementation. It has been suggested that all types of leucocyte, including lymphocytes, become saturated at vitamin C intakes of 100-200 mg per day (Levine *et al.* 1996). Therefore, it is likely that lymphocytes were already saturated prior to supplementation in the present study, and that increasing the availability of vitamin C led to no further uptake by these cells. However, compartments such as the plasma do not become saturated until consumption

is considerably greater than that in the normal diet of the subjects in the present study (Hornig *et al.* 1980; Levine *et al.* 1996; Benzie and Strain 1997). This may be an important factor, since certain cells (e.g. erythrocytes) accumulate vitamin C in proportion to the amount in extracellular fluid (Evans *et al.* 1982; May *et al.* 1995). It is not clear whether skeletal muscle accumulates vitamin C in proportion to the concentrations in the surrounding medium, or whether muscle more closely reflects leucocyte concentrations. However, Voskoboinik *et al.* (1998) observed that incubated smooth muscle cells accumulated vitamin C in proportion to the dose over the range 50-500 $\mu mol.l^{-1}$. These authors noted, however, that it took several hours for intracellular differences to reflect extracellular availability.

The increase in circulating muscle proteins (creatine kinase and myoglobin) observed in the present study is consistent with findings in earlier studies (Chapters 4 and 5). Creatine kinase activity showed a delayed peak the day after exercise, whereas myoglobin concentrations peaked 1h after exercise. This temporal discrepancy between these two markers of muscle damage has been reported following several forms of exercise (Goodman *et al.* 1997; Sorichter *et al.* 1997; Koller *et al.* 1998), and may be related to different rates of release or clearance of these proteins. The results of the present study suggest that myoglobin is released more rapidly than CK, which may be due to the considerable size difference between these molecules. Furthermore, myoglobin is cleared within a few minutes from the circulation (Klocke *et al.* 1982), whereas the half-life for CK is approximately 15 hours (Neumeier *et al.* 1981). Consequently, it is unlikely that supplementation would have had any effect on myoglobin levels within 1h of exercise, or CK activity on the day of exercise. Nevertheless, since myoglobin concentrations remained elevated until 24h post-exercise, and CK until 72h post-exercise, there was probably sufficient time for these parameters to be modified by supplementation. Therefore, the similar levels of blood-borne muscle proteins indicates that the administration of vitamin C was unable to affect membrane permeability at any point.

Theoretically, the loss of sarcolemmal integrity may be due to extensive lipid peroxidation of membrane unsaturated fatty acids (Demopoulos 1973a). Lipid

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peroxidation may also decrease membrane fluidity, and therefore alter membrane structure and function without the loss of membrane integrity (Demopoulos 1973a). In the present study, plasma malondialdehyde (MDA) was elevated until 24h after exercise in both placebo and vitamin C groups. This indicates that lipid peroxidation continued until this time, as MDA is cleared from the circulation within several hours (Jenkins *et al.* 1993).

It has been suggested that the best measure of muscle damage is not a biochemical marker such as CK, but the functional assessment of maximal voluntary force (Warren *et al.* 1999). Contractile dysfunction may highlight damage inside cells that is not reflected by the extent of lipid peroxidation or the efflux of muscle proteins (Kong *et al.* 1994; Van Der Meulen *et al.* 1997). Both supplemented and placebo groups exhibited poorer muscle function until 72h after exercise, and the decline was most prominent in the leg flexors. This is in agreement with earlier reports that this muscle group is the most affected after this type of exercise (Chapter 4). After certain forms of extremely damaging exercise, the loss of force may persist for several weeks (Pearce *et al.* 1998). A decline in the ability to generate force may be largely the result of damage produced by severe physical stress (Stauber 1989; Armstrong *et al.* 1991). Alternatively, impaired muscle function may occur partly as a consequence of free-radical reactions (Zerba *et al.* 1990b). The results of the present investigation suggest that if the disturbance to muscle function was mediated by radicals generated in the post-exercise period, supplementation with vitamin C appeared to be unable to alter the extent of the decline.

Secondary damage may be the result of the non-specific actions of phagocytes during an inflammatory response (Weiss 1989; Pyne 1994; Smith 1994; MacIntyre *et al.* 1996). The prolonged efflux of muscle proteins in the present study may indicate that the damage process continued in the post-exercise period. Circulating levels of cytokines increase during inflammation, including after damaging exercise (Pedersen *et al.* 1998). There were problems reliably detecting IL-6 in plasma (Appendix 14), and several authors have reported peak concentrations after extremely damaging exercise to be only slightly above the lower limit of detection for the assay used in the present study (Bruunsgaard *et al.* 1997; Hellsten *et al.* 1997). Nevertheless, there was a very modest

significant increase in plasma concentrations of IL-1β after exercise, which is in agreement with some (Bury *et al.* 1996; Ostrowski *et al.* 1998b), but not all studies (Castell *et al.* 1997; Nehlsen-Cannarella *et al.* 1997; Ostrowski *et al.* 1998a). Cytokines found in the circulation after damaging exercise are believed to be derived from the site of inflammation within damaged muscle (Ostrowski *et al.* 1998b). However, detection may be difficult since they are largely local mediators of an inflammatory response (Pedersen *et al.* 1998). Consequently, the absence of cytokines in the circulation is not necessarily indicative of the absence of inflammation. However, the sensation of soreness is possibly an indirect indication that inflammatory processes were active in damaged muscle in the present study (Smith 1991).

Secondary damage may also be related to increased superoxide production by endothelial cell xanthine oxidase (Duarte *et al.* 1993; Radak *et al.* 1995). Damaged muscle exhibits increased expression of xanthine oxidase for several days after eccentric exercise (Hellsten *et al.* 1997). Furthermore, Crenshaw *et al.* (1993) reported that the greatest damage after prolonged exercise was in muscle fibres adjacent to capillaries. Uric acid concentrations were elevated until 24h after exercise in the present study, and therefore there may have been proportional superoxide production. Alternatively, elevated uric acid concentrations may be the product of xanthine dehydrogenase, which would not produce the superoxide radical (Sjödin *et al.* 1990). The source of hypoxanthine required for this reaction in resting individuals the day after exercise is unclear, although it may suggest a disturbance to normal resting metabolism following damaging exercise.

Three members of the placebo group had particularly low soreness ratings, which largely explains the lower soreness observed in this group. These subjects perceived that they had received the vitamin C treatment, and it is possible that they under-reported the extent of pain. Furthermore, their other results were not consistent with such low responses, and at least one of these subjects had difficulty walking normally because of severe soreness. Consequently, this circumstantial evidence may suggest that analysis of these results ought to be taken no further, as subjects had inaccurately attempted to gauge which treatment they had received. Alternatively, since pain is

influenced to some degree by expectations (O'Connor and Cook 1999), it may simply reflect the fact that these subjects were less sore than they had anticipated.

In spite of the likely explanations for the apparently greater soreness in the vitamin C group discussed above, it may be prudent to consider other potential explanations. Activated neutrophils appear to accumulate vitamin C very rapidly, possibly to protect against self-inflicted oxidative damage (Washko *et al.* 1993). Furthermore, it appears that supplementation with high doses of vitamin C may improve neutrophil chemotaxis (Anderson *et al.* 1980; Anderson 1981). In theory, therefore, there may be greater numbers of neutrophils recruited to damaged muscle in the supplemented group, and they may survive longer due to the increased availability of vitamin C. However, there are a number of findings in the literature which suggest that any alteration in neutrophil function would have only beneficial outcomes. Supplementation with vitamin C appears to decrease the radical signal during the neutrophil respiratory burst in vitamin C deficient pigs (Schwager and Schulze 1998). Furthermore, supplementation in humans has been shown to reduce the activity of the neutrophil myeloperoxidase system (Anderson 1981). Although these observations were made in isolated cells studied *in vitro*, it may suggest that there would be less damage to healthy tissues in supplemented individuals. Furthermore, greater phagocyte activity in the supplemented group would be expected to be reflected in other markers of muscle damage. However, it is possible that the pain sensation is relatively independent of the extent of tissue damage (O'Connor and Cook 1999). Nevertheless, if such an effect was to be predicted, soreness might be expected to be lower after supplementation, since increased levels of vitamin C are believed to increase the degradation of histamine, a potent algesic (Johnston *et al.* 1992a; Johnston *et al.* 1992b).

In summary, the results of the present investigation demonstrate no benefit from the administration of vitamin C after damaging exercise. It is possible that inflammation is initiated in order to facilitate tissue healing (Tidball 1995), and plays no part in the secondary damage process. Similarly, xanthine oxidase-derived free-radicals may not contribute to muscle damage. However, earlier studies have suggested that this is not the case, as anti-inflammatory agents (Hasson *et al.* 1993; Bushell *et al.* 1996), and

inhibitors of xanthine oxidase (Chambers *et al.* 1985; Duarte *et al.* 1993; Radak *et al.* 1995), appear to confer distinct benefits. A more probable explanation is that consumption of vitamin C solely after exercise is unable to deliver this antioxidant to the appropriate sites with sufficient expediency. Although plasma concentrations of vitamin C increased very rapidly upon completion of exercise in the supplemented group, radical-mediated damage processes may have been well underway.

CHAPTER SEVEN

MUSCLE SORENESS AND DAMAGE AFTER PROLONGED SHUTTLE-RUNNING FOLLOWING TWO
WEEKS OF VITAMIN C SUPPLEMENTATION

7.1 Introduction

Although acute vitamin C supplementation potentially offered a great deal of
practicality, it was of no physiological benefit to exercise-induced muscle soreness and
damage (Chapters 5 and 6). It is possible that short-term supplementation immediately
before and after exercise fails to provide vitamin C at the times it would be most
advantageous. Acute supplementation prior to exercise resulted in a transient increase
in plasma vitamin C, and values had returned to baseline the day after exercise (Chapter
5). On the other hand, post-exercise supplementation may not have provided vitamin C
rapidly enough upon the completion of exercise to be effective as an antioxidant
(Chapter 6). In order to provide vitamin C at more appropriate times, an alternative
supplementation strategy may be required, possibly involving prolonged
supplementation.

Earlier studies in this thesis focused on vitamin C because, as a water-soluble
antioxidant, concentrations change rapidly following consumption (Chapters 5 and 6).
However, it is worth giving consideration to the relative benefits of other antioxidants
as part of a longer term period of supplementation. There is substantial evidence
supporting the use of vitamin E in the prevention of muscle damage and lipid
peroxidation (Dillard *et al.* 1978; Sumida *et al.* 1989; McBride *et al.* 1998), and
therefore this would appear to be an appropriate alternative. However, vitamin E has
been described as the last line of defence against lipid peroxidation (Niki 1996), and the
optimal strategy may be to prevent radicals from reaching lipid compartments (Frei *et
al.* 1989). Furthermore, vitamin E may stabilise membranes in a manner that is
independent of its antioxidant properties (Kagan *et al.* 1989; Niki 1996), and therefore

results of such studies would be difficult to attribute to free-radical reactions. Finally, although vitamin E has been repeatedly shown to protect membranes (Jackson *et al.* 1983; Van Der Meulen *et al.* 1997; McBride *et al.* 1998), it does not appear to affect ultrastructural damage or the ability to generate force (Warren *et al.* 1992; Jakeman and Maxwell 1993; Van Der Meulen *et al.* 1997). Although the maintenance of plasma membrane integrity is vitally important in terms of cell survival, the protection of intracellular structures is also of great importance. Vitamin C appears to offer considerable protection against ultrastructural damage following reperfusion injury (Bushell *et al.* 1996), and the decline in the ability to generate force after eccentric exercise (Jakeman and Maxwell 1993). Furthermore, vitamin C may also offer protection to hydrophobic compartments (Frei *et al.* 1990), including cell membranes. Therefore, the aim of the present study was to assess the effect of prolonged vitamin C supplementation on muscle damage and soreness after a damaging bout of exercise.

7.2 Subjects and methods

Sixteen male students volunteered to take part in this study, and were allocated to either a vitamin C or placebo group in a matched-group design (Table 7.1). Subjects who smoked or took vitamin supplements were excluded from the study. All participants were habitually active in a variety of sports, although none were familiar with the exercise protocol used in the present investigation.

	Age (years)	Height (cm)	Weight (kg)	$\dot{V}O_2$ max $(ml.kg^{-1}.min^{-1})$	Skinfolds (mm)	Weekly exercise sessions	Daily vitamin C intake (mg)
VC	24.7 ± 1.8	178 ± 2	75.6 ± 2.2	54 ± 1	38.8 ± 2.9	3 ± 1	147 ± 25
P	23.2 ± 1.7	180 ± 1	77.7 ± 1.9	52 ± 2	38.6 ± 4.5	4 ± 1	122 ± 29

Table 7.1   Subject characteristics in vitamin C (VC) and placebo (P) groups. Values for each group represent means ± SEM (n=8).

Experimental Design and procedures

Subjects performed the Loughborough Intermittent Shuttle Test (LIST) on only one occasion (Chapter 3.4), in order to ensure that the exercise remained unaccustomed for all participants. Fourteen days prior to performance of the LIST, subjects reported to the laboratory in a fasted state. A venous blood sample was taken, and subjects began the course of supplementation (Figure 7.1). On the first day (day 0), all subjects in the vitamin C group were given a 1g dose of vitamin C dissolved in 500ml of an energy-free version of a commercially available drink (Lucozade-Sport, SmithKline Beecham, UK), whereas subjects in the placebo group received the drink alone. This was partly to ensure that subjects in the vitamin C group commenced supplementation at a similar status, but also to investigate if there was any effect of acute supplementation on muscle function. There did not appear to be any such effect in the subset of subjects (n=6) who performed the muscle function test on these 2 days (Appendix 13). In order to maintain familiarity with the muscle function test, subjects also repeated the test on day 7 or 8 of the supplementation period. Supplements were given to subjects in the form of identical gelatin capsules, each containing 200mg of vitamin C (ascorbic acid) or 200mg lactose (Nova Laboratories Ltd., UK). Capsules were taken for 12 days in the morning and evening of each day (400mg d^{-1}), starting on day 1. Many studies fail to report the time the last dose was consumed (e.g. Kaminski and Boal 1992; Jakeman and Maxwell 1993), and therefore findings may be attributable to either the last dose or the extended period of supplementation. In the present study, supplementation was stopped approximately 36h before performance of the LIST, in order to ensure that acute and prolonged effects were separated. Therefore, the total amount of vitamin C consumed, above that received in the normal diet, was 5800mg.
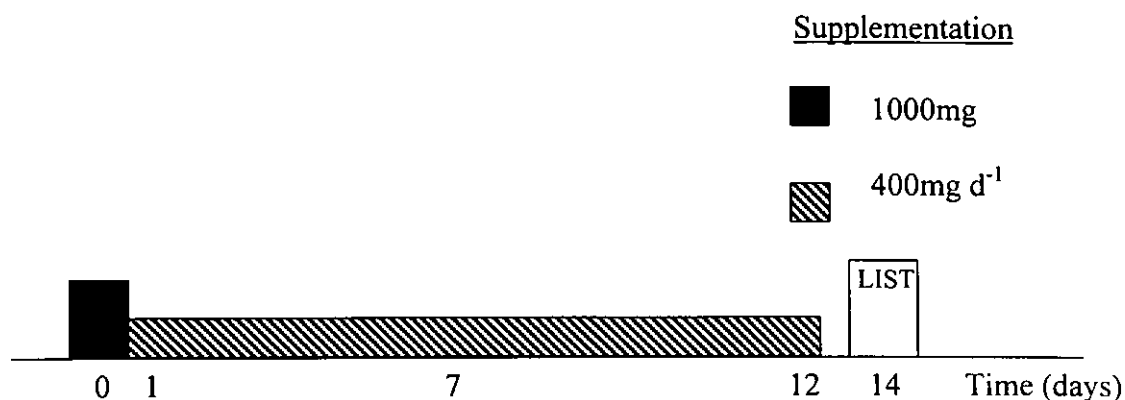
Figure 7.1   Prolonged supplementation prior to performance of the Loughborough Intermittent Shuttle Test (LIST). Subjects in the vitamin C group consumed vitamin C in the amount indicated per day, while subjects in the control group consumed an equivalent placebo (see text).

On the day of the test subjects arrived at the laboratory after an overnight fast of at least 10 h. A resting blood sample was taken using an indwelling cannula (Chapter 3.8), after which subjects consumed the light meal (Chapter 3.7) and rested for 2 h. The muscle function test (Chapter 3.6) was performed before exercise, immediately after exercise, and 2h after exercise. For 3 days after the LIST, subjects returned to the laboratory at approximately the same time of day ($\pm$ 1h) and following an overnight fast. A blood sample was taken from a forearm vein after subjects had been standing for at least 15 min. Subsequently, subjects rated the intensity of soreness and performed the muscle function test described earlier (Chapters 3.5 and 3.6). Over the 2 week period of supplementation, and over the 5 days of the main test, subjects were requested to consume a certain number of foods estimated to provide approximately 100mg of vitamin C per day (Appendix 4). Excluding this restriction, subjects were allowed to consume any other foods they wished. Over the 5 day period of the main test, subjects weighed and recorded their food and fluid intake. They were also instructed to abstain from strenuous exercise for 2 days prior to the LIST, and not to resume exercising until the conclusion of testing. Upon completion of the experiment, subjects were asked to consider whether they had an impression of which group they had been assigned (vitamin C or placebo), or if they had no perception of which group they had been allocated.

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Serum was analysed for CK and AST activities, and also for uric acid, cortisol, IL-6 and CRP concentrations (Chapter 3.9). Plasma was analysed for vitamin C and malondialdehyde concentrations, and lymphocytes for vitamin C concentrations (Chapter 3.9). Additionally, whole-blood was used in the determination of haemoglobin and haematocrit values, and also lactate and glucose concentrations (Chapter 3.9).

Statistical analysis

An independent two-way analysis of variance with repeated measures was used to compare results between treatments and over time. Where significant F ratios were found, a Tukey test was used to determine the cause of the variance. Certain results were not normally distributed (CK, myoglobin and CRP), and therefore these values were log transformed prior to ANOVA. Log transformation always resulted in a normal distribution, and therefore these ANOVA results are reported. When there were only single comparisons, a Student's t-test for correlated data was used to determine whether any differences between treatments existed. Significance was accepted at the 5% level. Values are presented as means $\pm$ standard error of the mean (SEM).

7.3 Results

The physiological responses to exercise were similar between groups. Heart rate throughout exercise was 171 ± 4 b. min^{-1} in the vitamin C group, and 169 ± 5 b. min^{-1} in the placebo group. Mean rating of perceived exertion was also very similar, being 15 $\pm$ 1 in both groups. Blood glucose concentrations at the end of exercise were 3.8 ± 0.1 mmol.l^{-1} in the vitamin C group, and 4.0 ± 0.1 mmol.l^{-1} in the placebo group. Post-exercise blood lactate concentrations were similar in vitamin C and placebo groups (3.9 ± 0.4 *vs.* 4.1 ± 0.3 mmol.l^{-1}). Mean ambient temperature in vitamin C and placebo groups was similar (14.9 ± 0.3 *vs.* 15.3 ± 0.3 °C), as was relative humidity (59 ± 3 *vs.* $63 \pm 3\%$). Subjects in the vitamin C group consumed 1.0 ± 0.1 l of water during exercise and lost 1.9 ± 0.1 kg in body mass (corrected for fluid intake), which was not different to the placebo group, which consumed 1.0 ± 0.1 l of water and lost 1.8 ± 0.1 kg in body mass. Mean sprint time over the course of the LIST was 2.53 ± 0.05 s in the vitamin C group, and 2.52 ± 0.07 s in the placebo group. Sprint times in sets 5 and 6

were longer than in set 1 in both groups (P<0.05). Plasma volume did not change over the period of the test in either vitamin C or placebo groups (Table 7.2).

	Baseline (-2h)	Pre	PE	1h PE	2h PE	24h PE	48h PE	72h PE
VC	------	2.0 ± 1.3	-3.3 ± 1.4	3.6 ± 2.5	0.9 ± 2.6	2.0 ± 1.7	1.3 ± 1.2	1.2 ± 1.2
P	------	0.2 ± 1.2	-5.5 ± 1.1	3.2 ± 1.9	1.5 ± 2.3	2.3 ± 2.8	1.1 ± 1.9	4.0 ± 2.4

Table 7.2   Estimated changes in plasma volume over the testing period in vitamin C (VC) and placebo (P) groups. Values in each group are means ± SEM (n = 8). Pre: pre-exercise, PE: post-exercise

There were no differences between groups in terms of dietary composition over the testing period (Table 7.3). Both groups consumed approximately 80mg of vitamin C per day using the dietary guidelines provided.

	Energy Intake (Kcal)	CHO (%)	Fat (%)	Protein (%)	Vitamin C (mg)	Vitamin E (mg)
VC	11.07 ± 0.67	53 ± 2	32 ± 2	15 ± 1	80 ± 13	7 ± 1
P	12.31 ± 1.02	54 ± 3	30 ± 3	15 ± 1	84 ± 13	7 ± 1

Table 7.3   Daily dietary composition assessed over the main test in vitamin C (VC) and placebo (P) groups. Values in each group are means ± SEM (n = 8). CHO: carbohydrate.

Plasma and lymphocyte vitamin C

Plasma vitamin C concentrations were similar in both groups prior to supplementation (Figure 7.2). Plasma levels increased to 60.8 ± 3.2 $\mu mol.l^{-1}$ in the vitamin C group after supplementation, which was greater than the value of 50.0 ± 6.0 $\mu mol.l^{-1}$ observed at the same point in the placebo group (P<0.05). Exercise increased plasma vitamin C

concentrations in both groups (P<0.01), although values were higher in the supplemented group at the end of exercise and 24h after exercise (P<0.01).

There was no difference in lymphocyte vitamin C concentrations between groups at any point throughout the testing period (Figure 7.2). There was a slight trend for values to be higher after supplementation in the vitamin C group, although this did not reach statistical significance. However, lymphocyte levels of vitamin C increased over the course of exercise, irrespective of treatment (P<0.05).
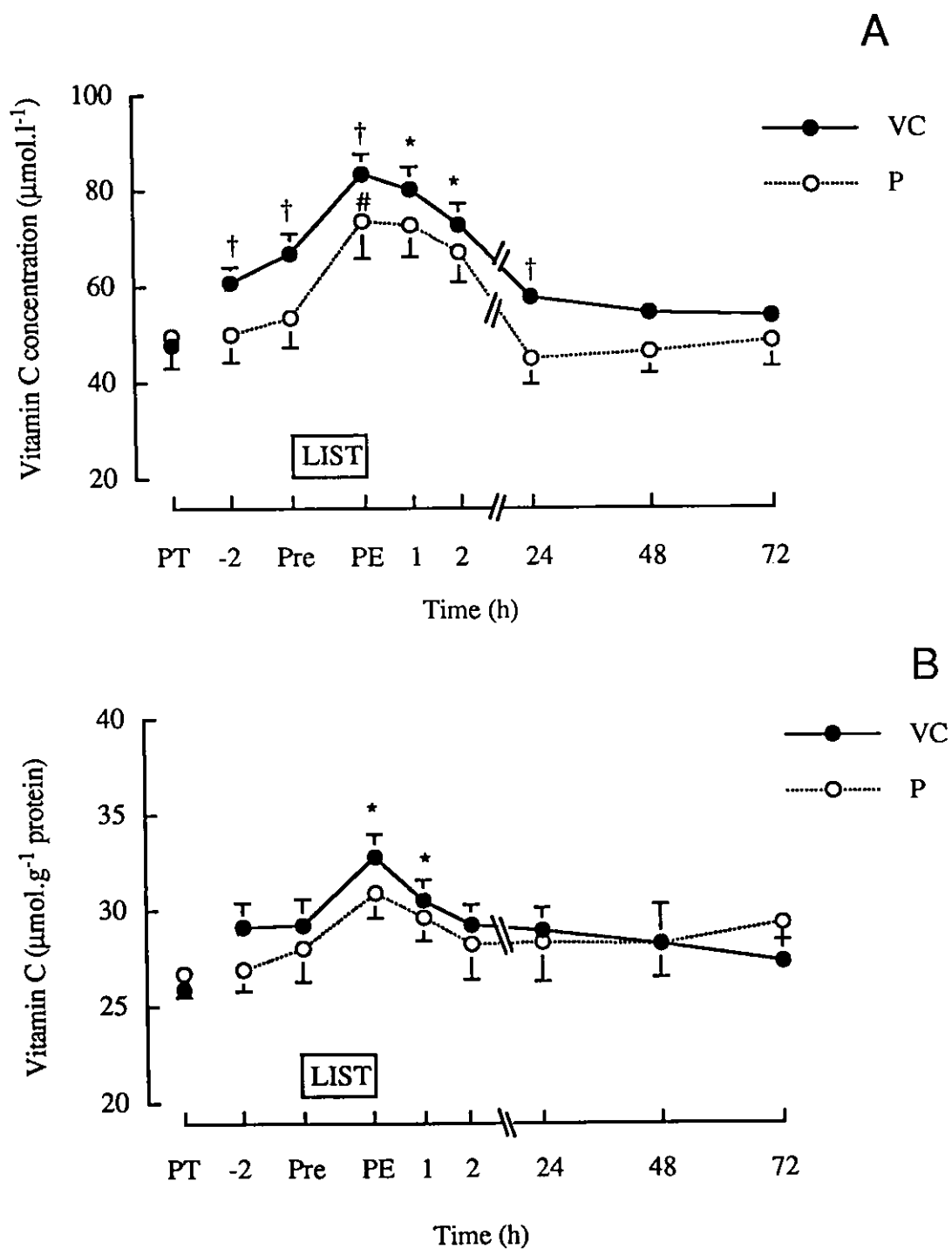
Figure 7.2   Plasma (A) and lymphocyte (B) vitamin C concentrations. Values represent means ± SEM (n=8). * Both groups above baseline values (P<0.01). † Vitamin C group above placebo and baseline values (P<0.01). # Placebo group above baseline values (P<0.01). VC: vitamin C, P: placebo, PT: pre-treatment, -2: baseline, Pre: pre-exercise, PE: post-exercise, LIST: Loughborough Intermittent Shuttle Test.

Markers of muscle damage

Serum creatine kinase activity increased after exercise in both groups (P<0.01), and remained above baseline values until 72h after the LIST (Figure 7.3). There was no difference between vitamin C and placebo groups at any point, with both groups showing peak activity approximately 24h after exercise. Serum myoglobin concentrations were elevated immediately after exercise (P<0.01), and reached a peak approximately 1h after the LIST in both groups (Figure 7.3). Myoglobin concentrations remained above baseline values until 24h after exercise (P<0.01), irrespective of treatment. Creatine kinase and myoglobin values were determined in a small subset of subjects 96h after the LIST (3 subjects from each group). Every subject had CK values lower than the previous day, and the mean for this group was 209 ± 36 U.l^{-1} (n=6). Myoglobin concentrations in this subset did not appear to change from the previous day, and the mean was 45 ± 2 µg.l^{-1} (n=6).

Plasma malondialdehyde

Plasma concentrations of malondialdehyde (MDA) increased after exercise in both groups, irrespective of treatment (Figure 7.4). Analysis of variance revealed a group-time interaction effect (P<0.05), although the *post-hoc* test was unable to detect any differences between vitamin C and placebo groups. However, plasma MDA concentrations remained above baseline until 24h after exercise in the placebo group (P<0.05), whereas MDA had returned to pre-exercise values 2h after the LIST in the vitamin C group.
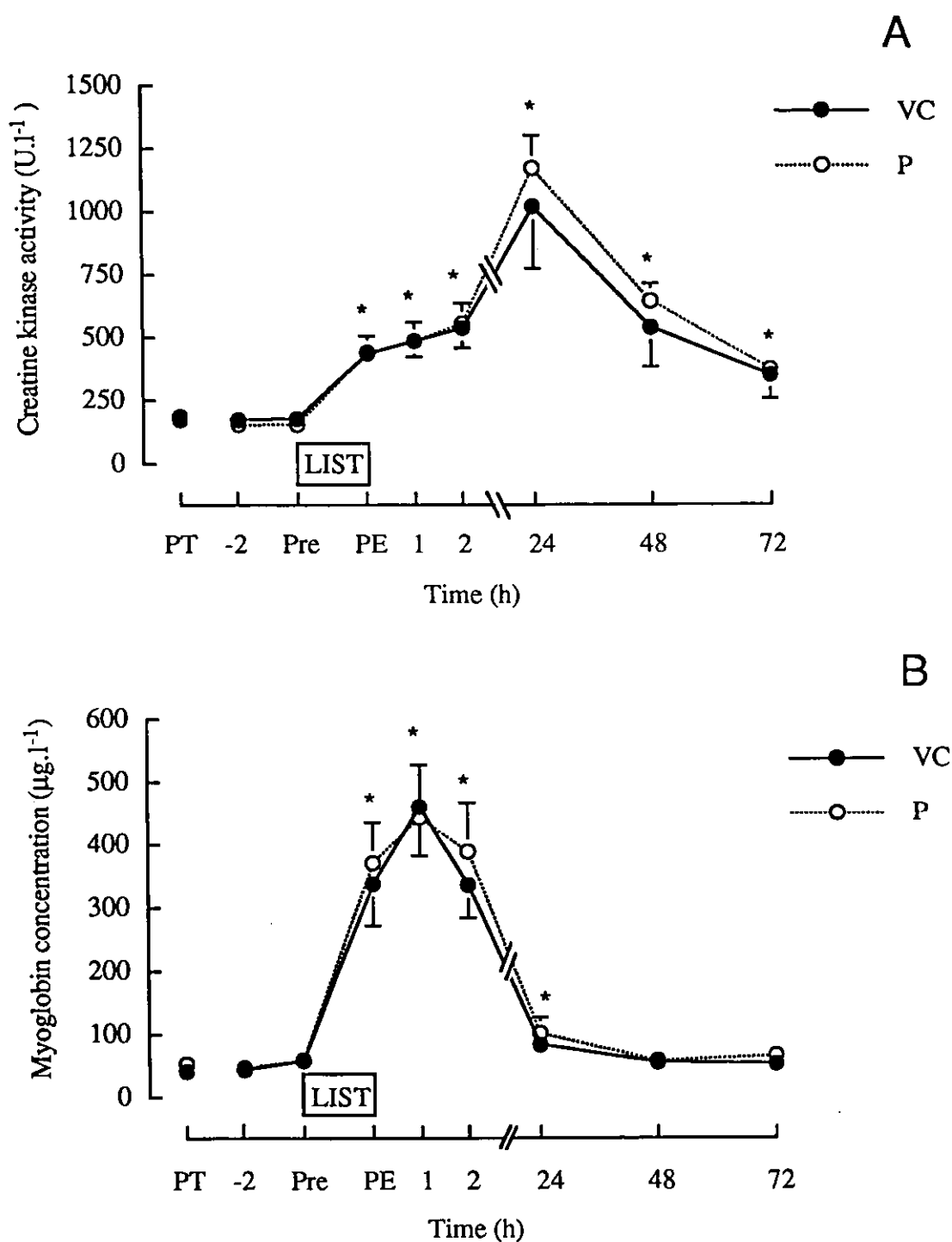
Figure 7.3   Serum creatine kinase activities (A) and myoglobin concentrations (B).
Values represent means ± SEM (n=8). * Both groups above baseline
values (P<0.01). VC: vitamin C, P: placebo, PT: pre-treatment, -2:
baseline, Pre: pre-exercise, PE: post-exercise, LIST: Loughborough
Intermittent Shuttle Test.

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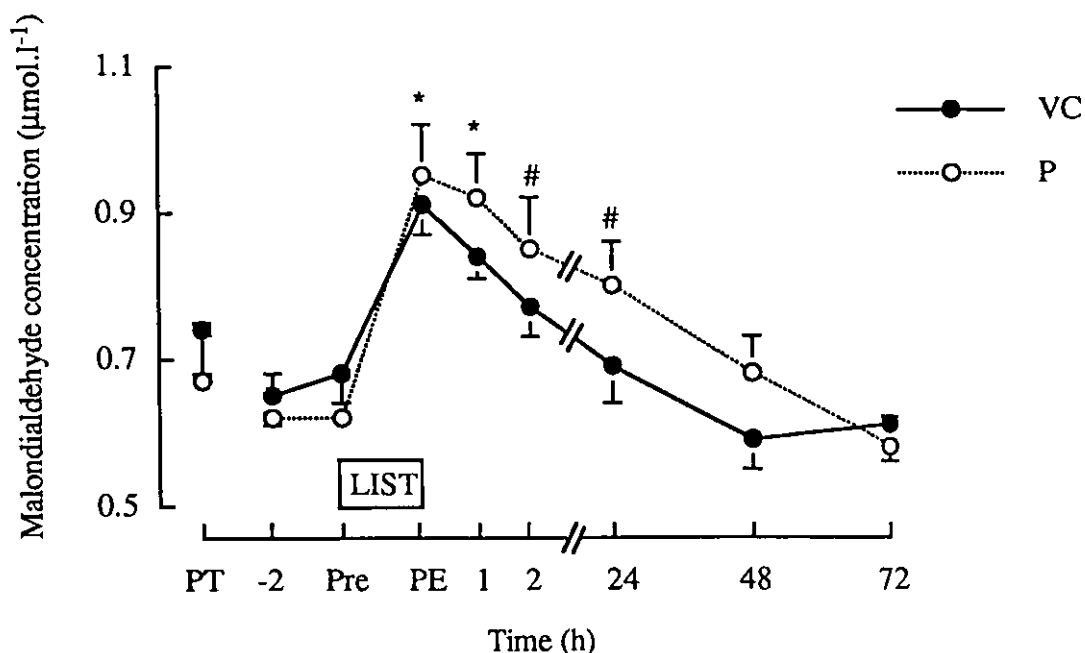
Figure 7.4   Plasma malondialdehyde concentrations. Values represent means ± SEM (n=8). * Both groups above baseline values (P<0.05), # Placebo group above baseline values (P<0.05). VC: vitamin C, P: placebo, PT: pre-treatment, -2: baseline, Pre: pre-exercise, PE: post-exercise, LIST: Loughborough Intermittent Shuttle Test.

Muscle function

Muscle function was impaired in the leg flexors and extensors of both groups after exercise (Tables 7.4 and 7.5). Leg extensor peak torque returned to pre-exercise values 48h after exercise, whether assessed during an isometric or isokinetic contraction. There was no difference in leg extension muscle function between vitamin C and placebo groups at any time. The leg flexors showed a similar decline after exercise, and muscle function was impaired up to 72h after exercise in the right leg (Table 7.4). Both the leg extensors and leg flexors in the placebo group showed a slight, non-significant, biphasic pattern for muscle function, particularly during isometric contractions. Peak torque was lowest immediately after exercise, recovering to some extent 2h later, and falling once more 24h after the LIST. The secondary decline in muscle function was less pronounced or absent in the vitamin C group, although values were only significantly above corresponding placebo values in the right flexor (P<0.05).

		Left Flexor						Right Flexor					
		Pre-exercise	Post-exercise	2h PE	24 h PE	48 h PE	72 h PE	Pre-exercise	Post-exercise	2h PE	24 h PE	48 h PE	72 h PE
Isometric	VC	100	85 ± 1 *	94 ± 3 *	92 ± 3 *	92 ± 3 *	96 ± 7	100	83 ± 3 *	90 ± 2 *	90 ± 2 * †	95 ± 3	101 ± 4
(%)	P	100	79 ± 4 *	86 ± 4 *	80 ± 5 *	84 ± 5 *	93 ± 2	100	78 ± 6 *	85 ± 4 *	76 ± 4 *	86 ± 6 *	86 ± 6 *
$60°s^{-1}$	VC	100	89 ± 3 *	88 ± 3 *	91 ± 2 *	93 ± 2 *	97 ± 2	100	86 ± 2 *	89 ± 2 *	91 ± 4 *	92 ± 3 *	95 ± 3 *
(%)	P	100	85 ± 4 *	89 ± 5 *	85 ± 4 *	92 ± 4 *	97 ± 3	100	79 ± 5 *	86 ± 4 *	82 ± 4 *	89 ± 4 *	90 ± 3 *
$180°s^{-1}$	VC	100	91 ± 3 *	90 ± 2 *	91 ± 3 *	94 ± 3	97 ± 3	100	89 ± 4 *	92 ± 1 *	92 ± 3 *	91 ± 2	95 ± 2
(%)	P	100	83 ± 3 *	88 ± 3 *	87 ± 4 *	93 ± 4	93 ± 2	100	84 ± 3 *	86 ± 4 *	86 ± 4 *	94 ± 2	94 ± 2

Table 7.4   Muscle function in vitamin C (VC) and placebo (P) groups in left and right flexors. Peak torque is expressed as a percentage of pre-exercise levels. Values are means ± SEM (n=8). * Below pre-exercise values (P<0.05).

		Left Extensor						Right Extensor					
		Pre-exercise	Post-exercise	2h PE	24 h PE	48 h PE	72 h PE	Pre-exercise	Post-exercise	2h PE	24 h PE	48 h PE	72 h PE
Isometric (%)	VC	100	89 ± 3 *	91 ± 2 *	93 ± 3 *	97 ± 3	103 ± 3	100	86 ± 5 *	92 ± 3 *	95 ± 3 *	99 ± 3	105 ± 3
	P	100	76 ± 4 *	90 ± 3 *	87 ± 4 *	95 ± 3	102 ± 2	100	83 ± 4 *	90 ± 4 *	88 ± 2 *	94 ± 4	102 ± 3
$60 \degree s^{-1}$ (%)	VC	100	92 ± 3 *	92 ± 3 *	94 ± 3 *	95 ± 3	102 ± 3	100	88 ± 2 *	92 ± 2 *	94 ± 3 *	98 ± 3	99 ± 2
	P	100	83 ± 3 *	91 ± 3 *	90 ± 3 *	100 ± 3	104 ± 4	100	84 ± 3 *	89 ± 3 *	88 ± 4 *	92 ± 2	96 ± 2
$180 \degree s^{-1}$ (%)	VC	100	93 ± 3 *	97 ± 3	98 ± 3	99 ± 3	103 ± 3	100	94 ± 2 *	96 ± 2	96 ± 3	95 ± 3	101 ± 2
	P	100	88 ± 3 *	97 ± 2	95 ± 3	100 ± 2	102 ± 2	100	90 ± 3 *	99 ± 1	94 ± 3	98 ± 2	100 ± 2

Table 7.5 Muscle function in vitamin C (VC) and placebo (P) groups in left and right extensors. Peak torque is expressed as a percentage of pre-exercise levels. Values are means ± SEM (n=8). * Below pre-exercise values (P<0.05).

Soreness

The muscle soreness results are shown in Tables 7.6 and 7.7. General whole-body soreness, and leg muscle soreness, were above baseline for at least 3 days after exercise (P<0.01). General soreness tended to be higher in the placebo group, although this difference did not reach statistical significance. However, the group-time interaction effect for general soreness using the 1-10 scale, and 0-100mm scale, revealed a trend (P=0.09 and P=0.1, respectively).

	Group	Pre-exercise	24h PE	48h PE	72h PE
GS	VC	1 ± 0	5 ± 0 *	4 ± 0 *	3 ± 0 *
(1-10)	P	1 ± 0	6 ± 1 *	5 ± 1 *	3 ± 0 *
GS	VC	3 ± 1	50 ± 6 *	33 ± 4 *	20 ± 5 *
(0-100)	P	5 ± 2	58 ± 6 *	47 ± 8 *	18 ± 4 *
HCS	VC	2 ± 0	5 ± 1 *	4 ± 1 *	3 ± 1 *
(1-10)	P	2 ± 0	6 ± 1 *	5 ± 1 *	3 ± 1 *
LES	VC	2 ± 0	6 ± 0 *	4 ± 1 *	3 ± 0 *
(1-10)	P	3 ± 0	5 ± 1 *	5 ± 1 *	3 ± 0 *

Table 7.6 General muscle soreness (GS) assessed on two different scales, and active soreness in individual muscle groups. HCS: hamstring curl soreness, LES: leg extension soreness. Values are means ± SEM (n=8). * Above pre-exercise values (P<0.01). VC: vitamin C, P: placebo, PE: post-exercise.

Upper-body muscle soreness is shown in Table 7.7. Soreness in these parts of the body was only elevated above baseline levels in the placebo group, and 24-48 h after exercise soreness was significantly higher than that observed in the vitamin C group (P<0.05).

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	Group	Pre-exercise	24h PE	48h PE	72h PE
BCS	VC	1 ± 0	2 ± 0	2 ± 0	1 ± 0
(1-10)	P	1 ± 0	4 ± 1 * †	3 ± 0 * †	2 ± 0
TES	VC	1 ± 0	2 ± 0	2 ± 0	2 ± 0
(1-10)	P	1 ± 0	3 ± 1 * †	4 ± 1 * †	2 ± 1
LPS	VC	1 ± 0	2 ± 0	2 ± 0	2 ± 0
(1-10)	P	1 ± 0	4 ± 1 * †	3 ± 0 * †	2 ± 1

Table 7.7   Active muscle soreness in individual parts of the body, assessed on 1-10 scale during each movement. BCS: biceps curl soreness, TES: triceps extension soreness, LPS: lateral pull soreness. Values are means ± SEM (n=8). * Above pre-exercise values (P<0.01), † Above the corresponding value in the vitamin C group (P<0.01). VC: vitamin C, P: placebo, PE: post-exercise

Interleukin-6 and C-reactive protein

Serum concentrations of interleukin-6 (IL-6) increased more than 8-fold as a result of exercise in both groups (P<0.01). In the placebo group, IL-6 increased further in the 1 h following exercise, whereas IL-6 concentrations in the vitamin C group declined over the same period (Figure 7.5). Two hours after exercise, IL-6 concentrations were significantly greater in the placebo group than in the vitamin C group (P<0.01).

Serum concentrations of C-reactive protein (CRP) were not different between groups at any time over the testing period (Figure 7.5). However, values were above immediate pre-exercise values both 24h and 48h after exercise (P<0.01). The highest CRP concentrations were seen 24h after exercise, which represented a 2-fold increase above pre-exercise values.
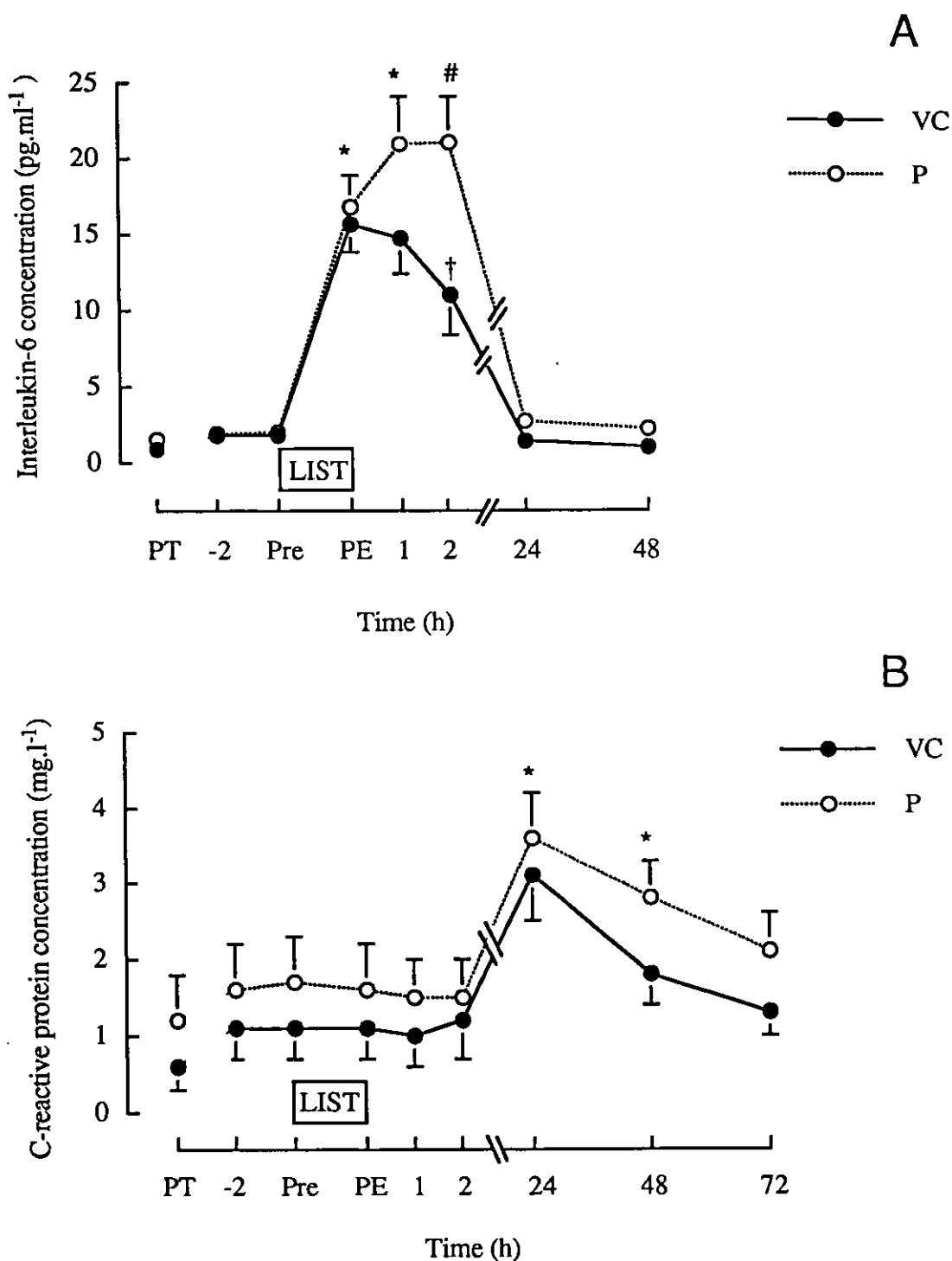
Figure 7.5   Serum concentrations of interleukin-6 (A) and C-reactive protein (B).
Values represent means ± SEM (n=8). * Both groups above baseline
values (P<0.01), # Placebo group above baseline values (P<0.01), †
Vitamin C group above baseline values but below placebo group values
(P<0.01). VC: vitamin C, P: placebo, PT: pre-treatment, -2: baseline,
Pre: pre-exercise, PE: post-exercise, LIST: Loughborough Intermittent
Shuttle Test.

		Pre-treatment	Baseline (-2h)	Pre-exercise	Post-exercise	1h PE	2h PE	24h PE	48h PE	72h PE
Cortisol (nmol.l^{-1})	VC	478 ± 38	533 ± 40	320 ± 31 *	630 ± 79 **	514 ± 64 **	407 ± 41	363 ± 41	439 ± 48	395 ± 48
	P	609 ± 49	608 ± 49	346 ± 30 *	627 ± 92 **	571 ± 69 **	398 ± 48	605 ± 39	518 ± 41	552 ± 32
Uric acid (μmol.l^{-1})	VC	340 ± 21	333 ± 21	326 ± 18 *	357 ± 25 *	365 ± 23 *	374 ± 22 *	347 ± 12 *	317 ± 12	311 ± 11
	P	328 ± 22	309 ± 15	311 ± 16 *	351 ± 15 *	349 ± 14 *	355 ± 15 *	333 ± 11 *	294 ± 15	305 ± 20

Table 7.8   Serum cortisol and uric acid concentrations before and after the LIST in vitamin C (VC) and placebo (P) groups. Values are means ± SEM (n=8) * Significantly different to baseline values (P<0.01) ** Significantly different to immediate pre-exercise values. PE: post-exercise.

Cortisol and uric acid

Serum cortisol concentrations fell in both groups after consuming the meal and resting for 2h (Table 7.8). Immediate post-exercise values were above immediate pre-exercise values in both groups ($P<0.01$), and remained above pre-exercise values 1h after exercise ($P<0.01$). The day after exercise, cortisol concentrations appeared to be lower in the vitamin C group, although the group-time interaction effect for the ANOVA was not statistically significant ($P=0.08$). There were no differences in serum concentrations of uric acid at any point between vitamin C and placebo groups (Table 7.8). Serum concentrations of uric acid increased in both groups after exercise ($P<0.05$), and remained above baseline values until 24h after exercise ($P<0.05$).

Subjective perception of group

The perceived treatment group of all sixteen subjects is shown in Table 7.9. The majority of subjects had not attempted to gauge which group they had been assigned, and had no impression of being in a particular group (n=10).

Subject perception of group	Vitamin C	Placebo
Perceived in VC group	1	1
Perceived in P group	3	1
No perception of group	4	6

Table 7.9   Perceived group (if any) at the end of the testing period. VC: vitamin C group, P: placebo group.

7.4 Discussion

The aim of the present study was to determine whether prolonged vitamin C supplementation would influence muscle damage and soreness following demanding exercise. The two week course of supplementation led to only a modest increase in plasma levels of vitamin C at the time of exercise. Nevertheless, there was some improvement to certain aspects of muscle soreness and function, although there was no effect on circulating markers of muscle damage.

Plasma vitamin C concentrations after supplementation were lower than might initially be anticipated with the dose used in the present study (Jakeman and Maxwell 1993; Levine et al. 1996). However, this probably reflects the 36h period between consumption of the last supplement and the start of exercise. Plasma vitamin C concentration in the supplemented group remained above that in the corresponding placebo group at the end of exercise and 24h later. However, there was no difference between treatment and placebo groups after this time, and therefore increased plasma vitamin C availability was relatively short-lived. Furthermore, supplementation had no effect on lymphocyte vitamin C concentrations, which is consistent with the findings in Chapter 6 and those reported by Levine et al. (1996). As discussed in the previous chapter, this is likely to reflect the subjects' relatively high vitamin C intake before the period of supplementation.

The presence of muscle proteins in the blood is widely accepted as an indication that muscle tissue has been damaged (Newham and Jones 1983; Evans 1987; Ebbeling and Clarkson 1989; Sorichter et al. 1997). The pattern and extent of muscle protein efflux in the present investigation was similar to that reported in earlier studies (Chapters 4 and 6). The peak values for blood-borne muscle proteins were probably largely related to damage which occurred during exercise. The delayed peaks may be partly attributable to the prolonged transit time of large muscle proteins (Volfinger et al. 1994), and the temporal discrepancy between peak myoglobin and creatine kinase may be a reflection of the relative half-lives of these proteins in the circulation (Neumeier et al. 1981; Klocke et al. 1982). There were no differences between groups for either

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myoglobin or creatine kinase, which suggests that the disruption to the sarcolemma was unaffected by supplementation. One explanation for increased membrane permeability is extensive lipid peroxidation of membrane unsaturated fatty acids (Demopoulos 1973, Jackson *et al.* 1995). Markers of lipid peroxidation have been reported to be elevated immediately after demanding exercise (Kanter *et al.* 1993; Child *et al.* 1998c), and several hours after damaging exercise (Maughan *et al.* 1989; McBride *et al.* 1998). In the present study, there was a similar increase in malondialdehyde at the end of exercise in vitamin C and placebo groups. Vitamin C is believed to regenerate membrane vitamin E, in addition to working independently in hydrophilic compartments (Packer *et al.* 1979; Niki 1987; Chan 1993). Consequently, during exercise, vitamin C was probably unable to scavenge radicals before they reacted with lipids, or to contribute to the recycling of vitamin E and other hydrophobic antioxidants within lipid structures.

Although vitamin C and placebo groups were very similar in many ways immediately after exercise, supplementation was associated with several beneficial changes after this point. Upper-body soreness was lower in the vitamin C group, and there was a trend for general soreness to be lower. These findings are similar to those reported after vitamin C supplementation for a shorter period, but with a higher dose (Kaminski and Boal 1992). Additionally, vitamin C appeared to modify the secondary decline in muscle force the day after exercise, although values were only significantly above corresponding placebo values in the right leg flexor. Jakeman and Maxwell (1993) reported similar results after a different form of exercise. Vitamin C supplementation at the same dose as used in the present study improved maximal voluntary force the day after exercise, but not immediately after exercise (Jakeman and Maxwell 1993). Therefore, vitamin C supplementation may influence both secondary damage and soreness that develop after the cessation of exercise. However, Jakeman and Maxwell (1993) also reported that vitamin C improved low-frequency fatigue immediately after exercise. Therefore, it is possible that vitamin C offered some protection during exercise in the present study, which was subsequently responsible for improved soreness and muscle force. However, if such a benefit existed immediately after exercise in the present study, the available evidence shows that it was not associated

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with improved muscle function, lipid peroxidation, or the efflux of muscle proteins at this point.

Exercise induced-muscle damage subsequently initiates certain aspects of the inflammatory response (Smith 1991). Peak circulating IL-6 concentrations are frequently observed immediately after exercise (Hellsten *et al.* 1997; Ostrowski *et al.* 1998a; Ostrowski *et al.* 1998b), although the peak may be delayed by several hours (MacIntyre *et al.* 1999). Serum concentrations of IL-6 increased immediately after exercise in the present study, and were not different between groups at this point. However, IL-6 continued to increase over the following hour in the placebo group, whereas the vitamin C group exhibited a decline over the same period. One explanation for this finding is reduced initial damage during exercise, and consequently, reduced inflammation. In contrast, earlier reports suggested that vitamin C had no effect on circulating levels of IL-6 after a 2.5h run (Nieman *et al.* 1997). The discrepancy between this study and the present investigation is not entirely clear, although it may be a consequence of the carbohydrate given during exercise by Nieman *et al.* (1997), which may have masked any possible effects from vitamin C (Nehlsen-Cannarella *et al.* 1997).

Reduced inflammation following exercise may explain many of the findings of the present study, although the differences between groups were not always statistically significant. Activated neutrophils and other phagocytes generate free-radicals, which are able to degrade healthy tissue as well as injured tissue (Weiss 1989; Smith 1994). Therefore, inflammation may be partly responsible for the prolonged elevation of malondialdehyde in the placebo group, whereas this was absent in the vitamin C group. In support of this notion, Vasankari *et al.* (1998) reported that conjugated dienes were lower 90min after exercise following vitamin C supplementation, but unaffected immediately after exercise. Furthermore, a reduction in inflammation may also explain the lower soreness in certain muscles of the vitamin C group (Smith 1991). Additionally, MacIntyre *et al.* (1996) suggested that a secondary decline in muscle function was the product of inflammation, as this coincided with the period of greatest neutrophil accumulation in damaged muscle. This may explain the tendency for muscle

function to be improved the day after exercise in the vitamin C group in the present study. However, inflammation is not the only source of free-radicals in the post-exercise period. Xanthine oxidase may contribute to oxidative stress and muscle damage after the cessation of a demanding bout of exercise (Duarte *et al.* 1993; Radak *et al.* 1995). The elevated uric acid concentrations observed in the present study may be interpreted as evidence that this process was activated in muscle tissue, although this system would not necessarily produce free-radicals (Sjödin *et al.* 1990). Therefore, it is not clear whether oxidative stress after exercise in the present study was due to radicals derived from xanthine oxidase or inflammation. It is possible that vitamin C prevented some initial damage, and therefore inflammation. Alternatively, vitamin C may have minimised damage from xanthine oxidase-derived radicals produced after the cessation of exercise.

Increased circulating IL-6 is one of the major factors responsible for the induction of acute-phase proteins (Sipe 1990; Kopf *et al.* 1994). There have been a number of reports that demanding exercise leads to increased levels of C-reactive protein (CRP) (Gleeson *et al.* 1995a; Castell *et al.* 1997). In the present study, serum concentrations of CRP peaked 24h after exercise, and remained above pre-exercise values 48h after exercise. However, although there was a tendency for serum concentrations of CRP to be lower in the vitamin C group, CRP did not significantly reflect the differences in IL-6 between groups. It has recently been suggested that IL-6 may not be a good marker of inflammation arising from exercise-induced muscle damage. Croiser *et al.* (1999) reported that levels of IL-6 were increased similarly after two bouts of eccentric exercise, whereas the efflux of muscle proteins and muscle soreness were only increased after the first bout of exercise. These authors suggested that another factor explains increased levels of IL-6 after a bout of demanding exercise, possibly some form of hormonal stimulus. Alternatively, it may be due to the complex relationship between cytokines and free-radicals. Cytokines are able to increase free-radical production in certain cells (e.g. neutrophils), although the reverse is also true, and increased intracellular levels of radicals may increase cytokine production (Clark *et al.* 1988; Grimble 1996). This seems to be related to the direct activation of the transcription factor responsible for cytokine production (Grimble 1996). Therefore, it is

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possible that lower levels of IL-6 after vitamin C supplementation are a reflection of altered intracellular oxidative stress in the cells responsible for the production of IL-6, rather than lower levels of inflammation in response to tissue damage. Theoretically, if this pattern was reflected across the cytokine cascade, then inflammation may be reduced, and explain many of the findings of the present study. However, such an effect would also be expected to alter levels of CRP accordingly, and therefore the cause of the discrepancy between IL-6 and CRP remains unclear.

A prolonged decline in muscle force is generally considered to reflect a functional impairment, because it persists beyond the sensation of soreness (Clarkson *et al.* 1992), and furthermore, superimposing electrical stimulation generates no improvement in force (Saxton and Donnelly 1996). Therefore, as discussed earlier, the trend for the muscle function to be improved the day after exercise in the vitamin C group may reflect the absence of secondary damage. However, a recent report found that electrical stimulation of sore muscles resulted in greater levels of force than during voluntary contractions, although this was not the case prior to exercise (Child *et al.* 1998a). Consequently, the sensation of pain may affect the generation of maximal voluntary force, and this may be partly responsible for the decline in muscle function in the days after exercise in the present study. Therefore, it is possible that muscle damage was unaffected by supplementation in the present study, and that reduced soreness was responsible for the moderately improved force in the vitamin C group. Such an effect would also explain the similar levels of circulating markers of muscle damage. However, this explanation seems unlikely, since the extent of leg muscle soreness did not appear to be affected by supplementation.

Vitamin C plays a major role in collagen synthesis (Pandipati *et al.* 1998), and therefore vitamin C supplementation may theoretically alter the quality of connective tissue. There have been reports suggesting that demanding exercise leads to increased connective tissue turnover (Abraham 1977; Brown *et al.* 1997a). Although it is unlikely that damage to this tissue would explain the efflux of muscle proteins and myofibre damage, soreness may be a product of damage to this tissue (Abraham 1977; Stauber *et al.* 1990). Theoretically, improved quality of connective tissue prior to exercise may

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explain some of the findings of the present investigation, and would be independent of any antioxidant role of vitamin C. However, it is unclear to what extent two weeks of vitamin C supplementation would affect mature collagen turnover, which has a reported half-life of several months (Kivirikko 1970).

In summary, vitamin C supplementation offered some very modest benefits to certain aspects of muscle soreness and function. These findings are supported by lower levels of interleukin-6, and a modest decline in the extent of lipid peroxidation following supplementation. However, it is noteworthy that many of the results were isolated to specific muscles and time-points, and there was no effect on the efflux of muscle proteins. Interestingly, these beneficial outcomes occurred despite the fact that there was only a relatively small change in the plasma concentration of vitamin C at the time of exercise.

CHAPTER EIGHT

GENERAL DISCUSSION

The cause of delayed-onset muscle soreness has been investigated for many years, although there have been very few recommendations for the treatment of this unwelcome condition. Muscle soreness is regarded as a direct product of muscle damage, as sore muscles show considerable disruption to muscle architecture (Friden *et al.* 1981; Newham *et al.* 1983b). Recent work has shown promising results following supplementation with a number of different antioxidants (Jakeman and Maxwell 1993; Krotkiewski *et al.* 1994; McBride et al. 1998), and therefore the scavenging of free-radicals appears to provide some protection against both muscle soreness and damage. In the past, a great deal of emphasis has been placed on vitamin E as a potential antioxidant (Jackson *et al.* 1983; Van Der Meulen *et al.* 1997; McBride *et al.* 1998). In contrast, Goldfarb (1999) recently highlighted that there is a paucity of research on vitamin C and muscle damage. Therefore, the studies reported in this thesis make a contribution towards greater understanding of the role of vitamin C as an antioxidant.

Investigators in the past have principally used eccentric exercise to induce muscle damage and soreness, such as downhill running (Fielding *et al.* 1993) and bench stepping (Newham *et al.* 1983b). However, it was the intention at the outset of this thesis to make the exercise model as representative of 'real life' activities as possible. Some of the most popular activities are examples of multiple-sprint sports (Williams 1990), and therefore this form of exercise would appear to provide a logical extension of earlier work on exercise-induced muscle soreness and damage. In the four studies reported in this thesis, the exercise protocol used was the Loughborough Intermittent Shuttle Test (LIST), which was designed to simulate multiple-sprint sports (Nicholas *et al.* 1995). The LIST led to a pronounced increase in soreness, and also an increase in circulating markers of muscle damage, lipid peroxidation, and inflammation. There was also a marked decline in muscle function, which persisted for 72h after exercise in certain muscle groups.

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A considerable obstacle to the investigation of muscle soreness and damage following unaccustomed exercise, is that such exercise is only unaccustomed to an individual on one occasion. A particular form of exercise when repeated within two to three weeks will not produce the same severity of response, and therefore a cross-over design is inappropriate. This form of diminished response to a particular form of exercise has been termed the repeated-bout effect (Byrnes *et al.* 1985), and presents a number of problems to the investigation of nutritional interventions into muscle soreness and damage. Indeed, the failure to account for this phenomenon has confounded earlier investigations (Corbett 1967; Kuipers *et al.* 1985). One solution may be to adopt a matched-group design, which was the approach used in most of the studies reported in this thesis (Chapters 4, 6 and 7), although this is not without its limitations. For example, there is considerable variation in factors such as serum creatine kinase activity and soreness following damaging exercise (Nosaka *et al.* 1996), and therefore it is difficult to ensure that groups are truly 'matched'. Alternatively, if the aim is to use the same subjects in a long-term cross-over design, then 8-10 weeks is needed in order to avoid the repeated-bout effect (Byrnes *et al.* 1985). Such a long period between trials not only presents practical problems, but also introduces other confounding factors such as seasonal variation in training and diet. An alternative approach may be to exercise one limb following some form of treatment, and allow the other limb to act as a control for the same subject. In many ways this may appear an ideal solution, although this approach also has a number of flaws. Exercising one limb is not only an unrealistic form of exercise, but also involves a relatively small muscle mass. This latter point may be particularly important, since responses may be difficult to detect at the whole-body level (e.g. in blood samples). Furthermore, opposite limbs may respond differently to the same exercise, and it may be particularly difficult to control for limb dominance. Finally, there must be sufficient washout time between trials in order to ensure that there is no effect from prior treatment, which may confound the results of subsequent trials. Therefore, the matched-group design was chosen for the studies reported in this thesis after careful consideration of all the confounding factors. Although this experimental approach is not a perfect solution, it may be the design that has the fewest and most tolerable disadvantages.

The investigations that are reported in Chapters 5 and 6 employed acute vitamin C supplementation, principally due to the potential practical application of this approach. Although short-term supplementation increased plasma concentrations of vitamin C substantially, there appeared to be no physiological benefits. Neither muscle damage nor soreness were improved following vitamin C supplementation in these studies (Chapters 5 and 6). It is possible that the timing of this acute antioxidant supplementation was inappropriate (Chapters 5 and 6). In addition, there appeared to be some negative consequences of acute vitamin C supplementation, which remain partially unresolved. Although a number of explanations were proposed for these findings, it is possible that they reflect some form of adverse reaction to acute supplementation. In contrast, prolonged supplementation was associated with a number of beneficial outcomes, including some modest benefits to muscle soreness and function (Chapter 7). The simplest explanation for this discrepancy is that prolonged vitamin C supplementation ensures that this antioxidant is available when, and where, it is required. Interestingly, the modest benefits reported in Chapter 7 demonstrate that plasma concentrations of vitamin C are not the critical factor. Furthermore, supplementation may be unnecessary, because with careful food selection, a daily vitamin C intake of 400mg is achievable in the normal diet. Interestingly, daily consumption of 400mg provides a similar amount of vitamin C per kg body mass as achieved by large animals which are able to synthesise this vitamin (Levine 1986). Although such a comparison proves little, it is tempting to suggest that elevated vitamin C consumption ought to be derived from a change in regular eating habits, to include more vitamin C-rich foods.

It was hypothesised at the outset of the studies reported in this thesis, that because soreness was a direct manifestation of muscle damage, it could only be reduced through a corresponding reduction in muscle damage. Furthermore, because free-radicals were proposed to be involved in the damage process, antioxidants may subsequently reduce muscle soreness. Plasma malondialdehyde was determined as a marker of free-radical activity in the last three studies of this thesis. Interestingly, vitamin C supplementation immediately before or after exercise had no effect on lipid peroxidation, as reflected by changes in malondialdehyde (Chapters 5 and 6). On the other hand, prolonged

supplementation resulted in a modest reduction in lipid peroxidation after the end of exercise (Chapter 7). Nevertheless, in contrast to expectations, a reduction in lipid peroxidation was not associated with a corresponding reduction in circulating markers of muscle damage in the same subjects (Chapter 7). Therefore, based on these findings, it would be reasonable to suggest that free-radical processes were not responsible for the efflux of muscle proteins observed after the LIST. However, paradoxically, there were significant correlations between malondialdehyde and blood-borne markers of muscle damage after exercise (Table 8.1). Although it is tempting to suggest that this relationship shows that free-radicals are partly responsible for muscle damage, these observations are not necessarily indicative of a causal relationship. Therefore, based on the available evidence, it appears likely that free-radicals were not responsible for the efflux of muscle proteins, and consequently, vitamin C was unable to exert any effect on this parameter. Nevertheless, there were some other modest benefits associated with vitamin C supplementation in the study reported in Chapter 7. However, it is noteworthy that these benefits all developed after exercise, and may represent an altered response to muscle damage, rather than altered muscle damage *per se*.

As suggested in the previous discussion, the relationship between muscle damage and soreness may not be as strong as imagined at the beginning of the studies reported in this thesis. Consequently, the original premise may have been somewhat inaccurate, and although muscle damage leads to muscle soreness, there is probably considerable independence between these factors. This would explain the lack of a relationship between muscle soreness and markers of muscle damage observed repeatedly throughout this thesis (Chapter 4 and Table 8.1), and by other authors (Newham *et al.* 1986; Maughan *et al.* 1989). However, there are several other explanations for this lack of agreement, and therefore the question of whether soreness is dependent principally on the extent of muscle damage remains unresolved. Soreness may largely reflect damage to connective tissue, and therefore would not be expected to mirror the release of cytosolic myofibre proteins (Abraham 1977; Brown *et al.* 1997a). With this in mind, at least one study has reported markers of collagen turnover to be related to muscle soreness (Abraham 1977), and this may prove a useful measurement for inclusion in the future. Furthermore, the intensity of soreness may be influenced by a wide range of

other factors, including personality, expectations, and previous experiences (MacIntyre *et al.* 1995; O'Connor and Cook 1999). One approach that may gain some insight into this particular aspect of muscle soreness, may be the inclusion of some form of pain-response test or questionnaire as a preliminary measurement. In addition to the complex nature of muscle soreness, there is the more established question mark surrounding the use of circulating muscle proteins as an index of muscle damage (Kuipers *et al.* 1985; Van Der Meulen *et al.* 1991). However, at the present time, there does not appear to be simple and readily accessible alternative, and recent evidence suggests that circulating levels of myofibrillar muscle proteins are a reasonable reflection of muscle damage (Sorichter *et al.* 1997; Koller *et al.* 1998). It is noteworthy that, although muscle soreness and markers of muscle damage were not related, different measurements of muscle soreness, and different markers of muscle damage, were always related (Table 8.1). Consequently, there is greater confidence in each respective measurement, and the weight of the available evidence suggests that although muscle damage produces muscle soreness, a direct causal relationship is an unsatisfactory and overly simplistic explanation. A more appropriate suggestion may be that muscle damage creates the appropriate environment for muscle soreness to develop. This hypothesis appears more logical with hindsight, since considerable muscle damage is present immediately after exercise, whereas muscle soreness takes time to develop. Furthermore, it incorporates the concept that soreness has many dimensions, and therefore may be somewhat amenable to manipulation.

	CK PE	CK 24h PE ‡	Mb 1h PE ‡	Mb 24h PE	GS 24h (1-10)	GS 24h (0-100)	GS 48h (1-10)	GS 48h (0-100)	MDA PE	MDA 24h PE	IL-6 PE †	IL-6 Peak †	CRP 24h †	CRP 48h †
CK PE	----	0.65 *	0.69 *	0.24	0.19	0.13	0.04	0.27	0.56 *	0.31	0.36	-0.21	-0.31	-0.18
CK 24h PE ‡		----	0.72 *	0.62 *	0.20	0.21	0.21	0.50 *	0.46	0.23	0.51	0.01	-0.45	-0.19
Mb 1h PE ‡			----	0.42	0.23	0.23	0.15	0.36	0.78 *	0.42	0.58	0.20	-0.66	-0.23
Mb 24h PE				----	0.29	0.36	0.45	0.60 *	0.22	-0.05	-0.05	-0.34	-0.18	-0.15
GS 24h (1-10)					----	0.95 *	0.84 *	0.80 *	0.09	0.04	-0.41	-0.51	0.47	0.50
GS 24h (0-100)						----	0.89 *	0.85 *	0.14	0.10	-0.41	-0.42	0.17	0.24
GS 48h (1-10)							----	0.93 *	0.14	0.09	-0.29	-0.43	0.25	0.24
GS 48h (0-100)								----	0.24	0.10	-0.09	-0.46	0.08	0.14
MDA PE									----	0.82 *	0.65	0.51	-0.66	-0.12
MDA 24h PE										----	0.63	0.68	-0.55	0.07
IL-6 PE †											----	0.77 *	-0.46	-0.28
IL-6 Peak †												----	-0.52	-0.21
CRP 24h †													----	0.63
CRP 48h †														----

Table 8.1   Pearson product moment correlation coefficients for the parameters indicated. Taken from the placebo groups in Chapters 6 and 7 (n=16). CK: creatine kinase, Mb: myoglobin, GS: general soreness, MDA: malondialdehyde, IL-6: interleukin-6, CRP: C-reactive protein, PE: post-exercise. * relationships statistically significant (P<0.05). † values only available for the study reported in Chapter 7 (n=8). ‡ peak values for all subjects were recorded at this point.

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Although largely conjectural, the previous discussion may explain many of the findings reported in Chapter 7. Soreness may have been modified without a corresponding reduction in muscle damage, and explain the similar levels of circulating muscle proteins. Furthermore, maximum voluntary force may not have been influenced by muscle damage, but may have been affected by muscle soreness (Child *et al.* 1998a). A possible mechanism to explain these findings may have been a reduction in inflammation, a hypothesis supported by lower plasma IL-6 concentrations following vitamin C supplementation. It is feasible that muscle damage is the somewhat inevitable consequence of certain forms of exercise, but the response to initial damage is by no means pre-determined. However, it should be pointed out that the response to initial damage may also include considerable further damage (Friden *et al.* 1983; Newham *et al.* 1983b). Therefore, vitamin C may have reduced the extent of secondary damage, and consequently, led to both diminished soreness and a reduced secondary decline in muscle function. Although there was no evidence from this thesis to support this notion, other investigators have shown that free-radicals play a pivotal role in secondary damage (Zerba *et al.* 1990b; Duarte *et al.* 1993). Therefore, this cannot be ruled out as a possibility, although if secondary damage was modified by vitamin C supplementation, it was in the absence of any change to circulating markers of muscle damage.

There may be some advantages to a reduction in soreness without a corresponding reduction in muscle damage. It is possible that muscle damage is essential in order for adaptive processes to confer greater resistance to subsequent exercise. Therefore, a reduction in damage may theoretically compromise the adaptive process, although at the present time there is no evidence to support this hypothesis. Interestingly, free-radicals are known to induce the expression of heat shock proteins, which offer resistance to subsequent stress (Kilgore *et al.* 1998). However, a reduction in damage from the same form of exercise lasts several weeks (Byrnes *et al.* 1985), whereas the upregulation of heat shock proteins lasts only hours or days (McArdle and Jackson 1996). Therefore, a reduction in the upregulation of heat shock proteins would only be of consequence to individuals regularly performing exercise of the same nature.

Nevertheless, the interaction between free-radicals and the protective effects of the heat shock proteins will undoubtedly produce exciting results in the future.

Interestingly, there was still considerable muscle damage and soreness after performance of the LIST for the third time, although there were some signs of adaptation following the third exposure to the LIST (Chapter 5). Other studies have shown that prolonged demanding running continues to produce muscle damage in well-trained individuals accustomed to a particular activity (Warhol *et al.* 1985; Rokitski *et al.* 1994a; Tsintzas *et al.* 1995; Ostrowski *et al.* 1998b). In contrast, repeated exposure to eccentric exercise does not produce muscle damage if performed within a few weeks of a previous bout of the same exercise (Byrnes *et al.* 1985; Brown *et al.* 1997b; Croisier *et al.* 1999). This suggests that the muscle damage processes may be different for these forms of activity. Consequently, although muscle damage after activities such as the LIST is less pronounced than after eccentric exercise, it may be of more importance, since it cannot be completely abolished with increased familiarity.

In the study reported in Chapter 7, there was a pronounced effect from vitamin C supplementation on upper-body soreness, and a trend for general soreness to be lower. However, leg soreness did not appear to be affected in the same subjects. This may be an interesting observation, since it suggests that in the muscles with the lowest soreness, vitamin C exerts the greatest effect. It is possible that the damage was so extensive in the leg muscles, that vitamin C was unable to offer increased protection. Alternatively, it may reflect the inability to perceive subtle changes in the intensity of severe muscle soreness (e.g. leg muscles), whereas differences in moderate soreness are more readily distinguished (e.g. upper-body muscles).

It is important to consider whether any of the effects attributed to the antioxidant role of vitamin C may have been due to some other confounding factor. Gleeson *et al.* (1987) reported a relationship between the increase in plasma vitamin C during exercise and the increase in cortisol ($r=0.89$), and it was suggested that elevated vitamin C may arise as a consequence of cortisol secretion. However, Kodama *et al.* (1994) suggested that the mechanism may be reversed, whereby vitamin C indirectly stimulates the release of

cortisol, through an increase in the secretion of adrenocorticotropic hormone (ACTH). According to this process, vitamin C may possess the ability to restrict or suppress excessive immune responses. Cortisol regulates many aspects of the immune system (Goldstein 1992), including the suppression of IL-6 (Papanicolaou *et al*. 1996), and prostaglandin synthesis (Norman and Litwack 1987). Theoretically, this mechanism may explain the lower levels of IL-6 and soreness following prolonged supplementation (Chapter 7). However, vitamin C supplementation did not increase levels of cortisol (Chapters 5-7), and somewhat paradoxically, there was a strong trend for cortisol to be lower the day after exercise following prolonged supplementation (Chapter 7). Furthermore, there was no relationship between post-exercise plasma vitamin C and cortisol in the placebo groups reported in Chapters 6 and 7 (n=16); whether assessed in absolute (r=-0.20), or relative (r=-0.09), terms. Interestingly, cortisol has been shown to be elevated 2 days after damaging exercise, possibly due to the pain experienced in sore muscles (Gleeson *et al*. 1995b). However, cortisol was not elevated in the placebo group the day after the LIST in Chapter 7, and therefore this does not explain the apparent differences in cortisol between groups. Furthermore, cortisol concentrations in all eight members of the supplemented group were considerably lower than their fasted pre-exercise values, whereas cortisol concentrations in the placebo group were similar at these points. Since the overriding stimulus for cortisol at this time ought to be metabolic, these results are difficult to explain. It is possible that the determination of free cortisol may have presented a clearer picture (Gray *et al*. 1993), since this is the biologically active form of the hormone.

In summary, muscle soreness and damage arise as a consequence of physical overexertion, including unaccustomed exercise. Because such exercise is often .unplanned, early studies in this thesis focused on supplementation at short-notice immediately before or after exercise (Chapters 5 and 6). In spite of the convenience of this approach, acute vitamin C supplementation did not appear to offer any protection from exercise-induced muscle damage and soreness. There are questions regarding the practicality of prolonged supplementation, as it may be more productive to undertake specific training over the same period of time. However, it is often impossible to anticipate the nature of future demanding exercise, and therefore to prepare for every

eventuality. Consequently, prolonged supplementation may still be desirable, in order to render a particularly demanding or novel form of exercise less damaging. There appeared to be modest benefits attached to prolonged vitamin C supplementation, although the improvements were largely of a small magnitude (Chapter 7). Nevertheless, these results appear promising, and provide some impetus to investigate the many other possible permutations of supplementation with vitamin C that were beyond the scope of this thesis.

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HEALTH SCREEN FOR STUDY VOLUNTEERS   Name ....

It is important that volunteers participating in research studies are currently in good health and have had no significant medical problems in the past. This is to ensure (i) their own continuing well-being and (ii) to avoid the possibility of individual health issues confounding study outcomes.

Please complete this brief questionnaire to confirm fitness to participate:

1. **At present,** do you have any health problem for which you are:
   - (a) on medication, prescribed or otherwise........................Yes ☐   No ☐
   - (b) attending your general practitioner.............................Yes ☐   No ☐
   - (c) on a hospital waiting list...Yes ☐   No ☐

2. **In the past two years,** have you had any illness which require you to:
   - (a) consult your GP ....Yes ☐   No ☐
   - (b) attend a hospital outpatient department.......................Yes ☐   No ☐
   - (c) be admitted to hospital ....Yes ☐   No ☐

3. **Have you ever** had any of the following:
   - (a) Convulsions/epilepsy..Yes ☐   No ☐
   - (b) Asthma ....Yes ☐   No ☐
   - (c) Eczema ....Yes ☐   No ☐
   - (d) Diabetes..Yes ☐   No ☐
   - (e) A blood disorder..Yes ☐   No ☐
   - (f) Head injury...Yes ☐   No ☐
   - (g) Digestive problems..Yes ☐   No ☐
   - (h) Heart problems ....Yes ☐   No ☐
   - (i) Problems with bones or joints ....Yes ☐   No ☐
   - (j) Disturbance of balance/co-ordination ....Yes ☐   No ☐
   - (k) Numbness in hands or feet ....Yes ☐   No ☐
   - (l) Disturbance of vision ....Yes ☐   No ☐
   - (m) Ear / hearing problems ....Yes ☐   No ☐
   - (n) Thyroid problems...Yes ☐   No ☐
   - (o) Kidney or liver problems...Yes ☐   No ☐

(p) An allergic reaction, eg., swelling or breathing difficulties

Yes ☐ No ☐

4. Has any, otherwise healthy, member of your family under the age
of 35 died suddenly during or soon after exercise? ....Yes ☐   No ☐

**If YES to any question, please describe briefly if you wish (e.g. to confirm problem
was/is short-lived, insignificant or well controlled.)** ....

Additional questions for female participants

    (a) are your periods normal/regular?................................Yes ☐   No ☐

    (b) are you on "the pill"?..Yes ☐   No ☐

    (c) could you be pregnant? ....Yes ☐   No ☐

    (d) are you taking hormone replacement therapy (HRT)?

Yes ☐   No ☐

Thank you for your cooperation!

Appendix 2 Muscle soreness location

A scaled-down version of the diagram used in the identification of muscle soreness. Subjects were given a highlighter and asked to label all sites where there was any degree of soreness. Modified from Seely *et al.* (1992).
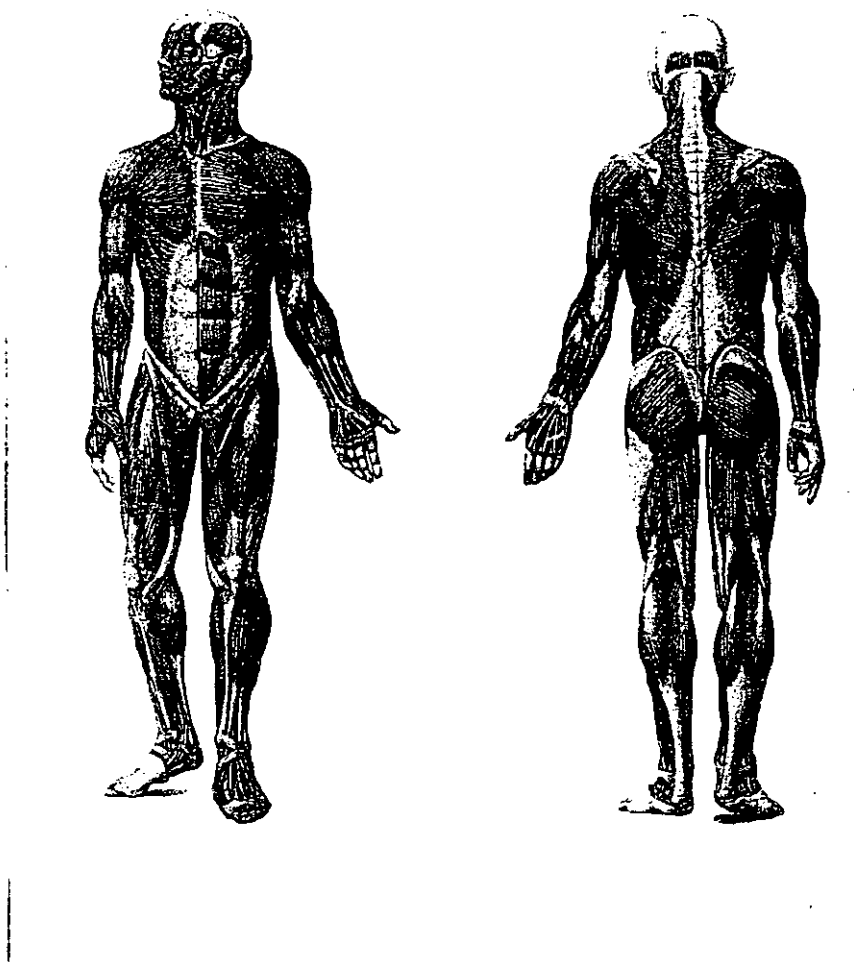
Appendix 3   **Muscle soreness intensity**

Scale A   *Scale B*

1 — Not Sore

2

3
A little Sore
                                    Normal

4

5
Quite Sore

6

7

8 — Very Sore

9 — Very very Sore

(shown horizontally)

10 — Very very Sore
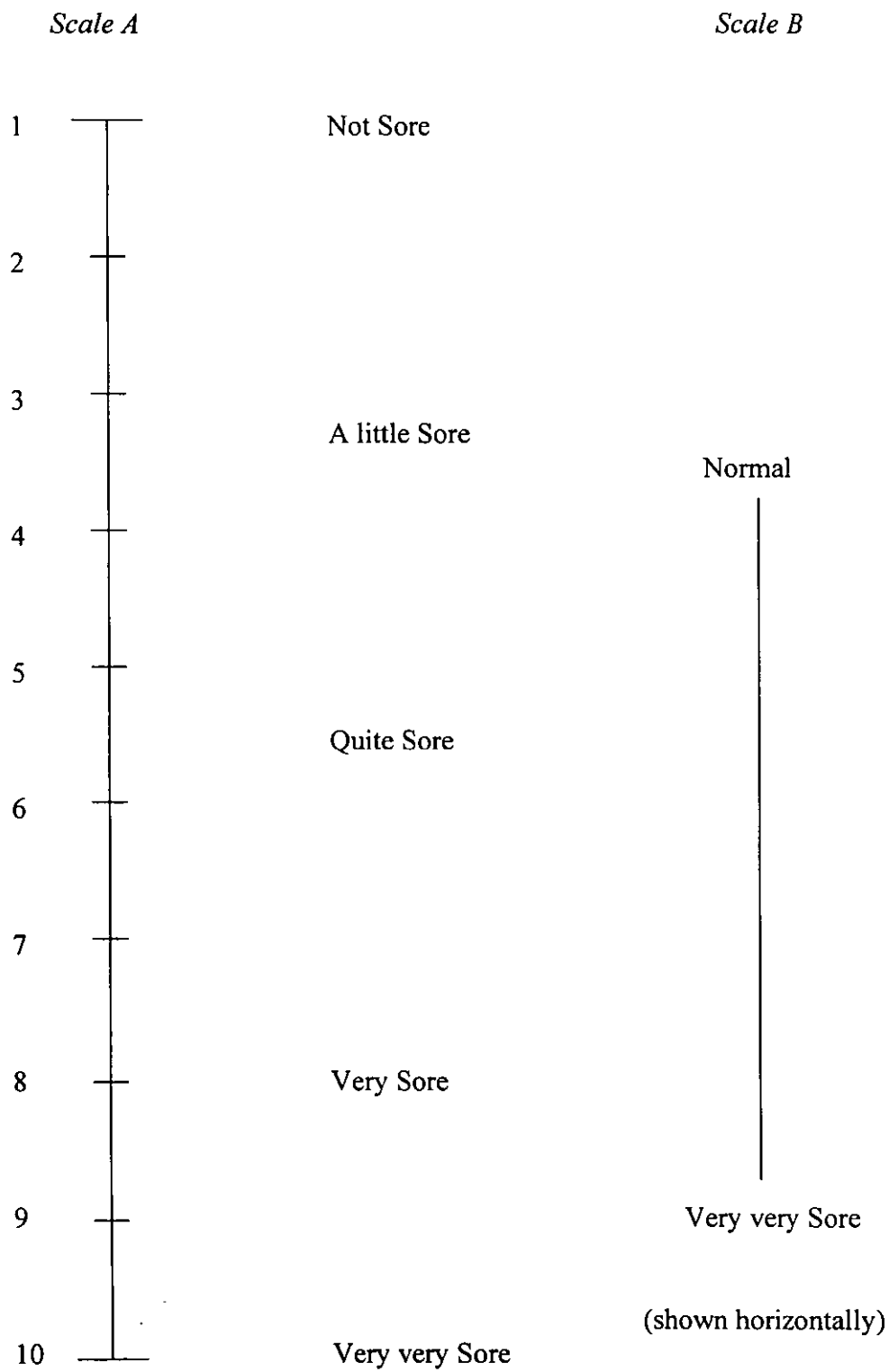
Appendix 4   Vitamin C portions

The following food portions contain 20 mg of Vitamin C. We would like you to try to eat 4 of these portions a day (no more than 5 portions). **You are allowed to eat as much of all other foods as you would like.** You do not have to record your diet over the 2 weeks of supplementation, but try and take in 4 portions of these foods. Over the period of the main trial you must record your diet and continue to stick to these guidelines.

50 ml ($\frac{1}{4}$ glass)	Orange juice (unsweetened)
250 ml (1 bottle)	Lucozade sport (NRG or Energy)
25 ml (5 teaspoons)	Ribena with 100 ml water ($\frac{1}{2}$ glass or carton)
80 ml (16 teaspoons)	Orange Squash with 320 ml water (2 glasses)
30 g ($\frac{1}{2}$)	Kiwi
55 g ($\frac{1}{2}$ small)	Grapefruit
100 g ($\frac{1}{2}$ small tin)	Mandarins tinned in juice/ fruit salad in juice
75 g ($\frac{1}{2}$ slice)	Canteloupe Melon (weighed without skin)
70 g (1 medium)	Tangerine/satsuma (weighed without skin)
15 g (1 $\frac{1}{2}$ slices)	Green, yellow or red pepper - raw
120 g (2 small)	Tomatoes
70 g (4)	Cherry Tomatoes
100 g (2 tablespoons)	Coleslaw
150 g (small)	Jacket potato
200 g (20)	Chips
200 g (4 small)	Roast/boiled potatoes
200 g (2 small)	Bananas
150 g (4 tablespoons)	Mixed Vegetables
45 g (Heaped Tablespoon)	Broccoli
75 g (2 tablespoons)	Cauliflower
30 g (3)	Sprouts

Appendix 5 **Lactate analysis**

Whole-blood lactate concentrations were determined according to a method modified from that described by Maughan (1982). This assay is based on the conversion of lactate to pyruvate in the presence of lactate dehydrogenase (LDH).

$$\text{Lactate} + \text{NAD}^+ \xrightarrow{\text{LDH}} \text{Pyruvate} + \text{NADH} + \text{H}^+$$

The amount of NADH produced is proportional to the amount of lactate present. The reaction is allowed to proceed by the removal of pyruvate due to the addition of hydrazine (to form pyruvate hydrazone).

Reagents and chemicals:
- 1.1 $mol.l^{-1}$ Hydrazine Buffer (pH 9.0)
- 70 $mmol.l^{-1}$ Hydrochloric acid (Lactate Diluent)
- 1.0 $mol.l^{-1}$ Lactate (Sigma Co., UK)
- NAD^+ (Boehringer Mannheim GmbH, Germany)
- Lactate dehydrogenase (Boehringer Mannheim GmbH, Germany)
- Sigma metabolite control (Sigma Co., UK) (Quality control)

Procedures
1. Standards were prepared using the 1.0 $mol.l^{-1}$ lactate solution in the range 0-10 $mmol.l^{-1}$., diluted to the same extent as the samples (1:10), and frozen until needed.

2. Frozen, deproteinised samples (plus standards and quality controls) were allowed to defrost at room temperature, and were subsequently mixed and centrifuged for 3min.

3. A reaction mixture was made, constituting 10μl LDH and 2mg NAD^+ per ml hydrazine buffer. A total of 200 μl of reaction mixture is required per tube.

4. Twenty (20) μl of supernatant was placed into a glass fluorimeter test tube.

5. Two hundred (200) μl of reaction mixture was added to the tube, mixed well, and allowed to incubate for 30-40 min.

6. One (1) ml of lactate diluent was added to each tube, and mixed well.

7. The fluoresence of the samples, quality controls, and standards were read using a fluorimeter (Model 8-9, Locarte, UK). The calibration range was established using the top and bottom standard.

8. The concentration in each sample was determined according to the linear regression equation established for the standards.

Appendix 6   **Vitamin E (α-tocopherol) analysis**

Plasma vitamin E concentrations were determined using high-performance liquid chromatography (HPLC) as described by Woodall *et al.* (1996). The only modification was that electrochemical detection was used in the place of spectrophotometric detection.

Chemicals (all from Sigma Chemical Co., UK, unless stated otherwise)
- Mobile phase = acetonitrile: methanol: chloroform (47:47:6 v:v) (BDH Laboratory Supplies, UK)
- Hexane (100%) (BDH Laboratory Supplies, UK)
- Ethanol (100%)
- α-tocopherol (100%)

Column and operating conditions
- Five (5) μm, 250mm * 4.6mm Spherisorb-5 ODS2 column (Technicol, UK).
- Electrochemical detection: Oxidising potential = 0.6 V (Range = 500nA/V).
- Flow rate was set at 2ml min^{-1}, which gave a retention time of approximately 6 min.

Procedures
1. Plasma samples were defrosted on the day of analysis.

2. Standards were prepared on a daily basis in the range 0-100 μmol.l^{-1}, by dissolving α-tocopherol in ethanol.

3. Three hundred (300) μl of distilled water was added to the standards.

4. A 300μl aliquot of plasma or standard was added to 200μl of hexane.

5. Samples and standards were vortex mixed for 2min, and then centrifuged at 4 °C for 5 min.

6. The supernatant was removed and 20 μl used for injection.

7. The concentration of α-tocopherol in the samples was determined as the area under the α-tocopherol peak in relation to a calibration curve determined for the standards. Values were corrected according to the extraction efficiency of each batch of analysis. The extraction efficiency was determined by adding a known amount of α-tocopherol to a standard and processing this as described above. The area of this measurement was related to a sample of the same concentration prepared in hexane and injected without prior extraction.

Appendix 7   Malondialdehyde analysis

Plasma malondialdehyde (MDA) was determined according to a modified method of Fukunaga *et al.* (1993). This method is able to distinguish the MDA peak from other interfering compounds (e.g. amino acids, bile pigments, haemoglobin, glucose).

Chemicals and solvents (all from Sigma Chemical Co., UK, unless stated otherwise)
- Mobile phase = acetonitrile: water (2:8 v:v) (BDH Laboratory Supplies, UK)
- 1-Butanol
- 2 M Sodium Acetate Buffer (pH 3.5). Made with Sodium Acetate Trihydrate, and concentrated hydrochloric acid to reduce the pH.
- 0.9% Sodium Chloride.
- Thiobarbituric acid (TBA).
- 5% Butyl Hydroxytoluene (BHT) made in 100% ethanol.

Column and Operating conditions
- Ten (10) μm, 250mm * 4.6mm techsphere ODS column (HPLC Technology, UK).
- Fluorimetric detection: Excitation at 515nm and emission at 550nm.
  Range = 0.02 RFU.
- Flow rate was set at 2ml min^{-1}, which gave a retention time of 1.5 min.

Procedures
1. Plasma samples were defrosted on ice on the day of analysis.

2. Standards were prepared with each batch of samples in the range 0-6 μmol.l^{-1}, using tetraethoxypropane (TEP) in 0.9% sodium chloride.

3. A 2% TBA in 2M sodium acetate solution was made (heated to dissolve) immediately before it was needed.

4. Twenty (20) μl of plasma or standards were added to eppendorf tubes. To this was added 180μl of 2M sodium acetate, and 3μl of 5% BHT. Finally, 20μl of 2% TBA solution was added to the tubes.

5. Tubes were mixed well and heated at 95 °C for 45 min. After heating, tubes were immediately cooled in ice.

6. Two hundred (200) μl of butanol was added, mixed well, and the tubes centrifuged for 5 min at 4 °C.

7. Fifty (50) μl of supernatant was used for injection.

8. The concentration of MDA in the samples was determined as the area under the MDA peak in relation to a calibration curve determined for the standards.

Appendix 8   Vitamin C analysis (Chapter 5)

Vitamin C analysis was performed using a modification of the method of Butcher (1991).

Chemicals (all from Sigma Chemical Co., UK, unless stated otherwise)
- Mobile phase = acetonitrile: 15 mmol.l^{-1} KH_2PO_4 water: glacial acetic acid (83.9:16:0.1 v:v) (BDH Laboratory Supplies, UK)
- Metaphosphoric acid (5%)
- Ascorbic acid

Column and Operating conditions
- Ten (10) μm, 250mm * 4.6mm Lichrosorb-NH_2 column (Fisons, UK).
- Electrochemical detection: Oxidising potential = 0.8 V (Range = 500nA/V).
- Flow rate was set at 2ml min^{-1}, which gave a retention time of approximately 5 min.

Procedures
1. Plasma and lymphocyte suspensions were defrosted on ice and kept chilled on the day of analysis (stored with 10% metaphosphoric acid 1:1 v:v - Chapter 3.8)

2. Standards were prepared on a daily basis in the range 0-300 μmol.l^{-1}, by dissolving ascorbic acid in 5% metaphosphoric acid.

3. Once samples had defrosted, they were mixed well and centrifuged at 4 °C for 10 min in order to obtain a clear supernatant.

4. Plasma samples and standards were diluted (1:4) in chilled 5% metaphosphoric acid, and 50μl used for injection. Lymphocyte samples and standards were not diluted, and 50μl of supernatant was directly injected.

5. The concentration of ascorbic acid in the samples was determined as the area under the ascorbic acid peak in relation to a calibration curve determined for the standards.

Plasma and lymphocyte vitamin C concentrations were determined using a method developed recently (S. Edmed, University of Surrey, personal communication).

Chemicals (all from Sigma Chemical Co., UK, unless otherwise stated)
- Degassed mobile phase = perchloric acid (Fisher Scientific, UK) adjusted to pH 1.5 at room temperature (pH reduced to 1.2 for plasma analysis).
- Metaphosphoric acid (5%)
- Ascorbic acid

Column and Operating conditions
- Five (5) μm, 250mm * 4.6mm C18 Luna column (Phenomenex, UK).
- Spectrophotometric detection: wavelength = 241 nm
- Flow rate was set at 1.2ml min^{-1}, which gave a retention time of approximately 3.4 min.

Procedures
1. Plasma and lymphocyte suspensions were defrosted on ice and kept chilled on the day of analysis (stored with 10% metaphosphoric acid 1:1 v:v - Chapter 3.8)

2. Standards were prepared on a daily basis in the range 0-300 μmol.l^{-1}, by dissolving ascorbic acid in 5% metaphosphoric acid.

3. Once samples had defrosted, they were mixed well and centrifuged at 4 °C for 10 min in order to obtain a clear supernatant.

4. Plasma samples and standards were diluted (1:1) in chilled 5% metaphosphoric acid, and 50μl used for injection. Lymphocyte samples and standards were not diluted, and 50μl of supernatant was directly injected.

5. The concentration of ascorbic acid in the samples was determined as the area under the ascorbic acid peak in relation to a calibration curve determined for the standards.

Appendix 10 **Dehydroascorbic acid analysis**

Measurement of plasma dehydroascorbic acid (DHAA) was undertaken in Chapter 6 according to a method based on the ascorbic acid analysis described in Appendix 9 (S. Edmed, University of Surrey, personal communication). The determination of DHAA was based on (1) determination of plasma ascorbic acid concentrations (AA), followed by (2) addition of a reducing agent to plasma (dithiothreitol, Sigma Chemical Co., UK) and subsequent determination of ascorbic acid concentrations (Total AA). Finally, (3) the amount of DHAA present in a sample was calculated (Total AA - AA).

Aliquots of plasma were collected and stored as described for vitamin C (Chapter 3.8), and defrosted on the day of analysis and kept chilled at all times. A 0.2% dithiothreitol (DTT) in 5% metaphosphoric acid solution was made, and 200 μl added to 200 μl of plasma supernatant and standards (mixed well). Ascorbic acid analysis was carried out as described above (Appendix 9), using both plasma samples and plasma samples treated with DTT. After mixing, samples treated with DTT were left to stand for at least 15 min prior to injection. Samples treated with DTT were not diluted, since the addition of DTT resulted in an equivalent dilution. Determination of Total AA and AA were carried out in the same batch of analysis. Retention times for ascorbic acid were identical after treatment with DTT, although it resulted in an extra DTT peak, which eluted approximately 12 min after injection. The results of this investigation are shown in Table A10.

Group	Subject	Analysis	-2	Pre	PE	1h	2h	24h	48h	72h
S	1	AA	59.8	55.4	72.6	109.4	89.7	69.2	70.3	69.2
		Total AA	60.5	54.2	74.8	109.5	88.7	65.3	72.1	71.1
S	2	AA	60.3	64.9	81.1	113.3	107.6	81.8	88.6	87.6
		Total AA	63.2	65.4	79.8	115.9	106.1	82.4	88.4	89.0
NS	3	AA	51.1	56.5	66.3	79.5	61.7	48.3	46.6	43.6
		Total AA	50.5	56.8	68.7	78.3	61.4	46.1	46.8	44.2
NS	4	AA	51.9	53.4	91.0	81.3	65.1	49.7	50.0	56.2
		Total AA	52.2	55.1	93.6	80.7	66.4	48.9	51.6	57.3

Table A10   Ascorbic acid (AA) and total ascorbic acid (ascorbic acid + dehydroascorbic acid) concentrations (μmol.l^{-1}) in four subjects over the time course of the study described in Chapter 6. Supplemented (S) and non-supplemented (NS) subjects. Time points: -2: baseline, Pre: pre-exercise, PE: post-exercise.

It appears that DHAA is absent from plasma, or in very low concentrations, since Total AA - AA did not consistently yield positive values for DHAA. These results confirm those of Dhariwal *et al.* (1991), and suggest that if DHAA is present in human plasma, it is less than 1-2 μmol.l^{-1}. Furthermore, it appears that neither supplementation nor exercise affected the circulating levels of DHAA.

The ingestion of an acute dose of vitamin C (ascorbic acid) was investigated in six male subjects over a period of 3.5h. The mean age, height and body mass of the subjects were 26.5 ± 1.4 years, 175 ± 3 cm, and 74.0 ± 3.7 kg, respectively. Subjects ingested a 1g dose of ascorbic acid (Roche UK Ltd., UK) with the standardised light meal (Chapter 3.7). Blood was drawn every 30 min through an indwelling cannula (Chapter 3.8), while the subjects rested in a seated position. The 1g of ascorbic acid was added to the drink immediately prior to consumption.

Blood was dispensed into a plain tube and left to clot for 30 min, and subsequently centrifuged at 3000 g to obtain serum. The remaining whole blood was dispensed into a lithium-heparinised tube, and used to determined changes in plasma volume using haemoglobin and haematocrit values (Chapter 3.9). Fresh serum was analysed within 3h of being collected for ascorbic acid concentrations using a commercially available spectrophotometric assay (Boehringer Mannheim GmbH, Germany). The CV for this assay was 6.8%. The results of this investigation are presented in Table A11. Results were analysed using a one-way ANOVA followed by a Tukey test.

	Baseline	0.5 h	1 h	1.5 h	2 h	2.5 h	3 h	3.5 h
Vitamin C (μmol.l^{-1})	54.5 (4.1)	61.5 (6.2)	77.9 (6.2) *	96.1 (8.7) *	100.2 (5.4) *	101.7 (6.7) *	99.2 (11.2) *	99.2 (10.0) *
Plasma Volume (%)	----	-1.8 (0.8)	-2.1 (1.6)	-2.2 (1.2)	-1.4 (1.0)	-0.4 (1.6)	-0.4 (1.7)	0.2 (1.6)

Table A11   Serum concentrations of vitamin C and percentage change in plasma volume after ingestion of 1g ascorbic acid with a light meal. Values are means (SEM) (n=6) * Significantly different to baseline values (P<0.05).

Appendix 12   **Leg flexor peak torque by trial-order (Chapter 5)**

		Left Flexor				Right Flexor			
		Pre - exercise	24 h PE	48 h PE	72 h PE	Pre - exercise	24 h PE	48 h PE	72 h PE
Isometric	T1	100	93 ± 5 *	92 ± 4	101 ± 4	100	91 ± 4 *	96 ± 3	101 ± 3
(%)	T2	100	89 ± 5 *	93 ± 5	92 ± 4	100	91 ± 4 *	94 ± 4	98 ± 2
$60°s^{-1}$	T1	100	95 ± 4	97 ± 3	103 ± 3	100	98 ± 3	100 ± 3	103 ± 2
(%)	T2	100	96 ± 3	94 ± 3	95 ± 3	100	97 ± 4	97 ± 3	100 ± 2
$180°s^{-1}$	T1	100	96 ± 3	97 ± 3	100 ± 3	100	96 ± 3	97 ± 2	97 ± 3
(%)	T2	100	98 ± 2	102 ± 2	98 ± 3	100	98 ± 1	96 ± 2	94 ± 2

Table A12 :   Muscle function during Trial 1 (T1) and Trial 2 (T2) in left and right flexors, irrespective of treatment.
Values are means ± SEM (n = 9). * Lower than pre-exercise values (P<0.05) PE: post-exercise.

Appendix 13 Peak torque in the leg flexors and extensors (Chapter 7)

After performing the muscle function test (Chapter 3.6), subjects in the vitamin C group consumed 1g of vitamin C dissolved in a drink (Chapter 3.7). The placebo group consumed an identical drink without vitamin C. The muscle function test was then repeated on day 2. Results were analysed using a paired t-test. There was no difference between values on day 1 and day 2 for either vitamin C or placebo groups. Therefore, acute ingestion of vitamin C in resting individuals does not appear to affect muscle function.

Muscle group	Group	Left		Right	
		Day 1	Day 2	Day 1	Day 2
Leg Flexors					
Isometric	VC	145 ± 7	140 ± 4	151 ± 6	145 ± 4
	P	151 ± 6	146 ± 9	136 ± 10	134 ± 11
$60\,°s^{-1}$	VC	129 ± 3	132 ± 8	130 ± 6	129 ± 5
	P	122 ± 7	125 ± 5	128 ± 5	131 ± 4
$180\,°s^{-1}$	VC	93 ± 2	92 ± 3	96 ± 4	90 ± 3
	P	91 ± 2	96 ± 4	96 ± 2	93 ± 2
Leg Extensors					
Isometric	VC	227 ± 9	229 ± 9	219 ± 9	222 ± 10
	P	251 ± 11	249 ± 11	237 ± 11	238 ± 11
$60\,°s^{-1}$	VC	188 ± 7	191 ± 8	190 ± 7	189 ± 11
	P	206 ± 10	211 ± 11	212 ± 9	215 ± 10
$180\,°s^{-1}$	VC	130 ± 6	128 ± 5	132 ± 4	128 ± 5
	P	146 ± 5	149 ± 5	148 ± 6	149 ± 6

Table A13   Peak torque (Nm) assessed on day 1 and day 2 of supplementation (Chapter 7). Values are means $\pm$ SEM. Values are for a subset of each group (n=6). VC: vitamin C group, P: placebo group.

Appendix 14   Interleukin-6 (Chapter 6)

Plasma concentrations of interleukin-6 (IL-6) were determined in Chapter 6 (Table A14). Interleukin-6 appeared to be absent from the plasma of most individuals, or in very low concentrations. The majority of values were below the detection limit of the assay used in this chapter (2 pg. ml^{-1}).

Subject code	Group	Baseline (-2h)	Pre-exercise	Post-exercise	1h PE	2h PE	24h PE	48h PE	72h PE
B	VC	<2	<2	<2	<2	<2	<2	<2	<2
D	VC	2.5	6	5.5	5.5	5.5	5.5	8	<2
E	VC	<2	<2	<2	<2	<2	<2	<2	<2
G	VC	<2	<2	<2	<2	<2	<2	<2	<2
I	VC	<2	<2	<2	<2	<2	<2	<2	<2
J	VC	3	<2	3	5	4	3	3.5	<2
M	VC	12.5	25	30	28.5	28	25	27.5	23
P	VC	26	22	24	23.5	20.5	20	21	15
A	P	<2	<2	<2	<2	<2	<2	<2	<2
F	P	2.5	<2	<2	<2	<2	13.5	8	<2
H	P	23	20	24	28	25	18	25	20
K	P	4	5.5	<2	<2	3.5	2.5	3	6.5
L	P	164	160	157	193	202	208	198	240
N	P	27.5	15	14	23.5	18	23	18	8
O	P	27	13.5	15	18	18	20.5	17.5	15
R	P	18	15	21	16.5	18.5	16.5	19	13

Table A14   Plasma interleukin-6 (IL-6) before and after the LIST (Chapter 6). Values are shown for each individual (pg. ml^{-1}).
VC: vitamin C group. P: placebo group.