Chapter One

Introduction: early epidemiological work

Physical activity is generally recognised as a therapeutic modality; the beneficial cardiovascular, respiratory and metabolic adaptations resulting from regular prolonged exercise are well recognised. However, the possibility that an improved level of physical conditioning augments immunological function, has only become the subject of serious scientific enquiry in the last three decades and has yielded conflicting results.

A dichotomy of responses has been reported. On the one hand, a high level of physical conditioning, has, in a few carefully controlled recent studies, provided evidence of a lower incidence of infection symptoms and beneficial effects on immune function which may increase resistance to infection (Nieman & Pedersen, 1999). Overtraining, and the combined psycho-physical stress of competitive endurance events and acute bouts of exhaustive endurance exercise, have, on the other hand, been associated with transient suppression of immune and host defences (König *et al.*, 2000).

Nieman (1994) initially proposed a "J" shaped model in explaining the paradoxical relationship between exercise and upper respiratory tract infection (URTI) risk. He postulated that whereas a moderate intensity and quantity of work over a prolonged

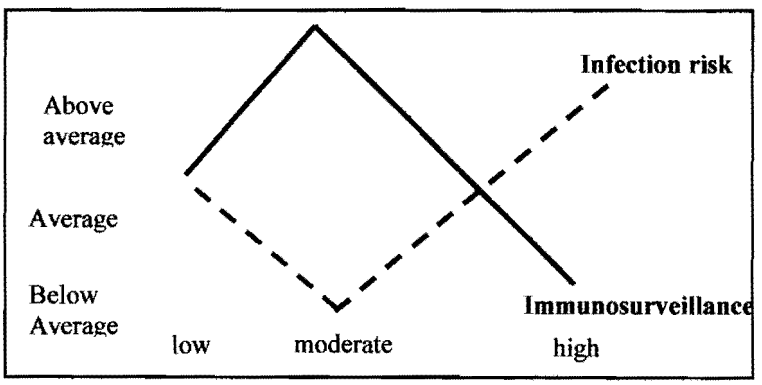

Figure 1.1: The paradoxical relationship between workload, risk of URTI & immunosurveillance athletes (adapted from Nieman, 2000)

period reduces the risk of infection below that of a sedentary individual, once a critical threshold is reached, the more strenuous the exercise, the greater the risk of infection. His recent adaptation of what has become a classic "J" shaped model, incorporates the effect which exercise workload has on immunosurveillance **(Figure 1.1).**

1.1 *Acute effects of competitive prolonged exercise*

Already in 1975, Ryan *et al.* reached the conclusion that "upper respiratory illness causes more disability among athletes than all other diseases combined." This was confirmed by Berglund & Hemmingson (1990) who reported that URTI was the most common reason for absence from training in elite skiers over a 12 month period **(Figure 1.2)**

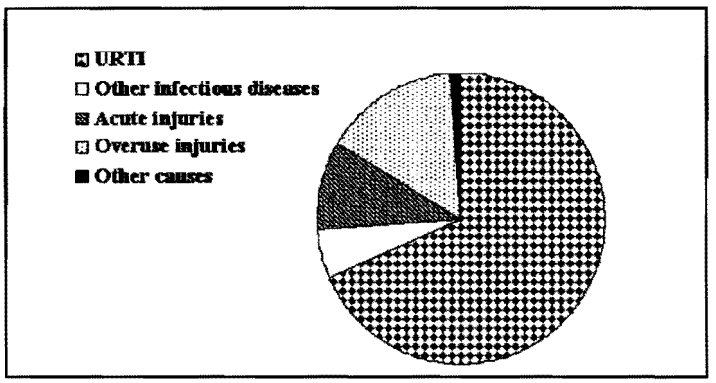

Figure 1.2: URTI, the main medical reason for absence from training in elite Swedish cross country skiers. Data from Berglund & Hemmingson (1990).

Several epidemiological surveys have subsequently confirmed clinical manifestation of immunosuppression in the form of increased incidence of URTI symptoms following participation in competitive marathon and ultramarathon running events (Peters & Bateman, 1983; Peters, 1990; Nieman *et al.*, 1989a; 1990a; Peters *et al.*, 1992; 1993; 1996) and during overtraining (König *et al.*, 2000).

In the first preliminary investigation (Peters & Bateman, 1983), a simple epidemiological survey was conducted on 150 successful finishers in the 1982 Two-Oceans Ultramarathon (56 km) run annually in Cape Town, South Africa, and their age-matched non-running controls who resided in the same households. Thirty-three percent of runners completing the race reported URTI symptoms during the two-week post-race period as opposed to 15.3% in the non-running control group. The

frequency of URTI symptoms was inversely related to the time taken complete the race ($p>0.01$) and almost half of the fastest runners experienced symptoms **(Figure 1.3).**

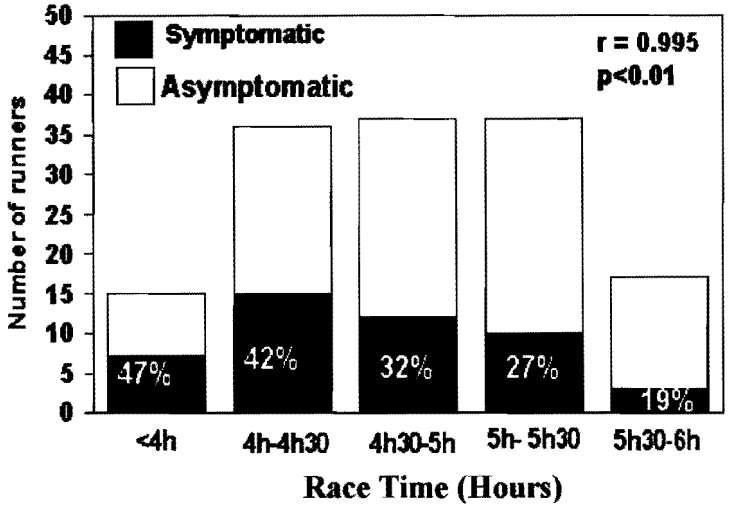

Figure 1.3: Distribution of symptomatic and asymptomatic runners according to time taken to complete the race (n=14; data from Peters & Bateman, 1983)

The finding that the fastest runners experienced the highest incidence of symptoms of infection, was supported by the observation that the runners completing the highest training weekly distance in preparation for the race, experienced the highest incidence of symptoms of infection. This greater incidence of symptoms of infection was attributed to (i) possible drying of the mucosal surfaces resulting from hyperventilation of cold, dry air and/or (ii) immuno-suppression resulting from elevated serum cortisol levels experienced during the race.

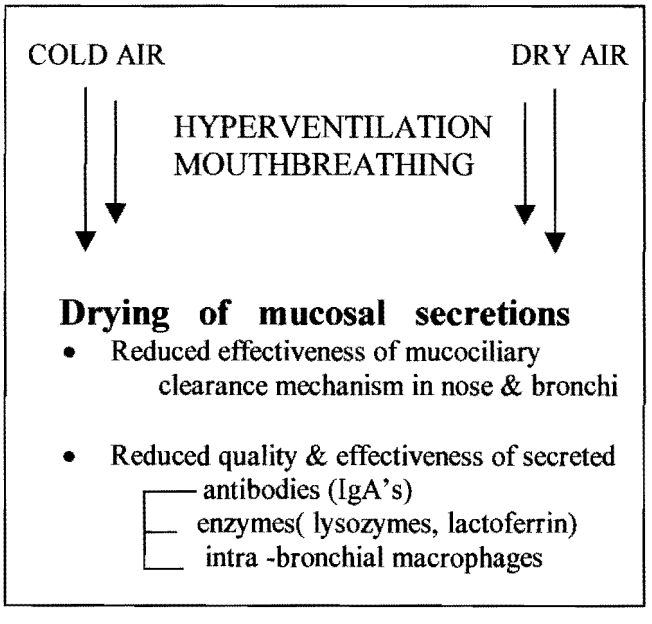

Figure 1.4: Possible mechanism by which distance running predisposes to increased incidence of URTI infection (Peters, 1990)

The finding of a significantly higher incidence of URTI symptoms among runners during the post-race period was confirmed by Peters in 1989 when the study was repeated at the Milo Korkie Marathon, a 56 km race taking place at an altitude of 1800 km above sea-level, between Pretoria and Johannesburg, South Africa. It was hypothesized that local mucosal damage resulting from hyperventilation and mouthbreathing (**Figure 1.4)** would have been greater at this altitude than at sea level due to the lower barometric pressure and concomitant reduction in relative humidity and thus would have resulted in a higher percentage incidence of URTI symptoms among runners competing over the same distance. This was not found in this sample of 108 runners surveyed in this study. A significantly higher incidence of symptoms of infection among the runners during the post-race fortnight than during matched, non-running controls, was, however, confirmed (**Figure 1.5)**.

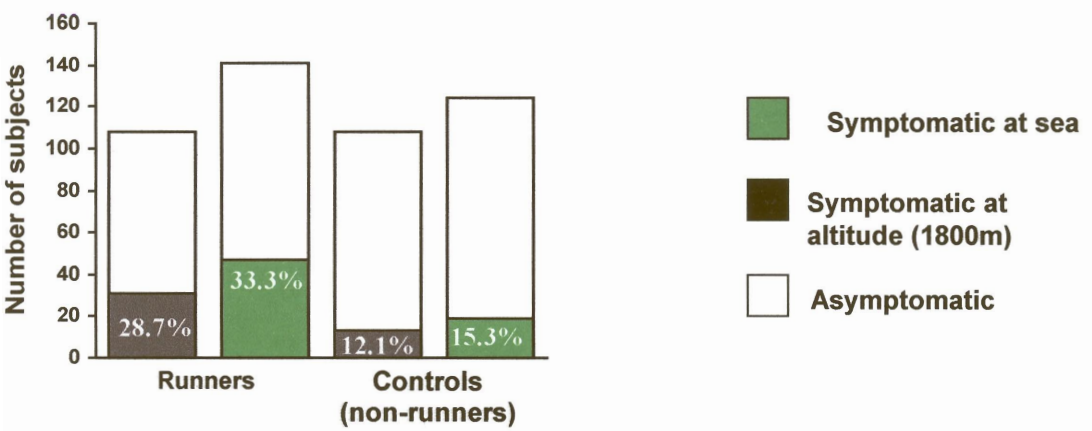

Figure 1.5. The incidence of URTI symptoms during the post- race fortnight in runners completing 56 km ultramarathons at sea level and moderate altitude and matched sedentary controls. Data from Peters & Bateman (1983) and Peters (1990).

In the same year a two-month investigation was conducted into the pre-and post-event incidence of URTI in a group of 273 participants in 5,10 and 20 km events in California (Nieman *et al.,* 1989a). While 25% of the runners training in excess of 25 km.wk^{-1} reported at least one episode of URTI during the two-month period, the incidence was higher (33,3%; p=0.09) in the less serious recreational runners completing less than 25 km.wk^{-1} in training. No increase in URTI incidence was reported in the runners during the 7-day post race period as compared to the incidence in the week prior to the race.

This study was followed by an investigation into the URTI symptom incidence in 2311 participants before and after the Los Angeles Marathon (LAM) [Nieman *et al.*, 1990a] which revealed an increase in odds ratio of infectious episodes (IE) with an increase in pre-race training distance ($p=0.04$). Reported incidence of illness was highest in those runners who completed > 97 $km.wk^{-1}$ while training in preparation for the event. Of the 1828 competitors in the Los Angeles Marathon without infectious episodes (IE) before the race, 12.9% reported IE during the week following the marathon vs. 2.2% in controls (well trained non-participating runners). These researchers concluded that runners may experience increased odds for IE during heavy training or following a marathon race.

Collectively, these independently conducted epidemiological surveys undertaken since 1983 provide consistent evidence of increased infection risk following acute intensive physical exertion.

1.2 *Anti-oxidant intervention studies*

Although the exact mechanisms underlying the increased URTI symptoms following participation in an ultramarathon were not known, I used the predictable increase in infection risk as a model to test the efficacy of anti-oxidant supplements in enhancing resistance to such infections.

I firstly conducted a double-blind, placebo-controlled study on runners and their matched non-running controls participating in the 1990 Comrades Marathon (Peters *et al.*, 1993). Supplementing with 600 mg vitamin C daily for the three-week period prior to the 90 km race resulted in the incidence of post-race URTI symptoms being more than halved in this group of Comrades runners when compared to the incidence amongst the runners receiving placebo. Thirty three percent of the vitamin C supplemented group reported the development of symptoms of URTI as opposed to an incidence of 68% in the runners receiving placebo. This supplementation did not, however, affect the incidence of URTI symptoms in the sedentary controls although previous findings of a significantly ($p<0.01$) shorter duration and a

subjective amelioration of the severity of the symptoms infections (Hemilä, 1992) was confirmed.

Further indirect evidence that the greatest benefit is to be derived from supplementary anti-oxidants was obtained from my findings at the 1991 Comrades Marathon. Supplementing with vitamin A, widely recognised for its anti-infective, rather than anti-oxidant properties (Bloem *et al.*, 1990; Coutsoudis *et al*, 1992), had an insignificant effect ($p<0.05$) on the incidence of URTI in the sample of runners studied (Peters *et al.*, 1992). Measures of blood vitamin A status revealed that neither runners receiving vitamin A supplementation nor runners on placebo were deficient in vitamin A **(Figure 1.6).**

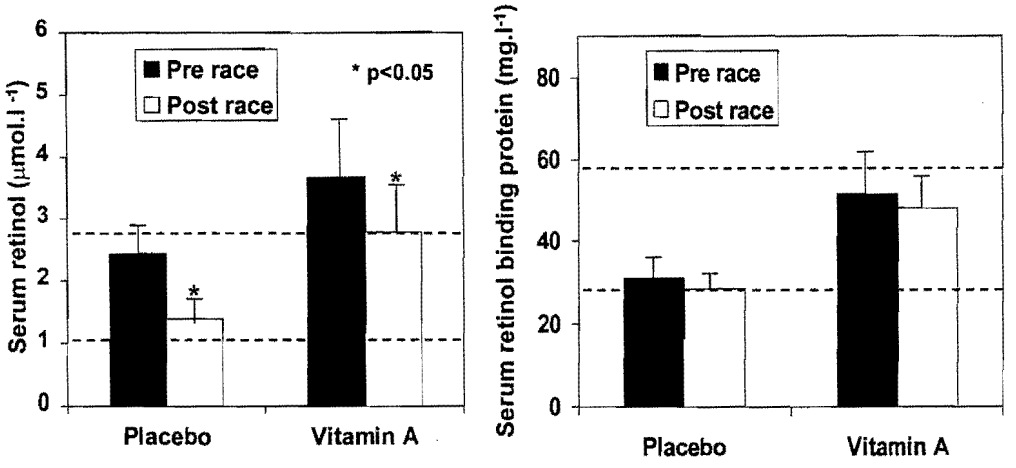

Figure 1.6. Mean (± SD) serum retinol and serum retinol binding protein concentrations in runners on placebo (n=11) and vitamin A supplementation (n=9) before and after the 1992 Comrades Marathon (Peters *et al.*, 1992).

Although both vitamin C and vitamin E have been shown to be of benefit when taken individually, several studies (Packer, 1986; Alescio *et al.*, 1992; Frei, 1994; Herbaczynska-Cedro *et al*, 1994, 1995) have shown that vitamin E in combination with Vitamin C is the most effective in terminating self-propagating free radical chain reactions. I therefore conducted another study in which the efficacy of three different anti-oxidant nutrient combinations were compared with respect to amelioration of URTI symptoms in ultramarathon athletes (Peters *et al.*, 1996).

Runners (n=178) and matched controls who resided with the runner (n=162) were divided into four treatment groups receiving daily supplementation with 500 mg of vitamin C, 400 IU vitamin E and 500 mg vitamin C, and 300 IU vitamin E, 300 mg vitamin C and 18 mg Beta (β) carotene or placebo for a three week period. The incidence of symptoms of URTI was monitored in both runners and controls during the two weeks following the ultramarathon. This was, once again, significantly greater in the runners on placebo than in the sedentary, non-running controls. Of the three groups of runners receiving anti-oxidant supplements, it was the group with the highest mean total (ie. in diet and supplements) daily vitamin C intake (1004 mg) who reported the lowest incidence of symptoms of upper respiratory tract infection **(Figure 1 .7).**

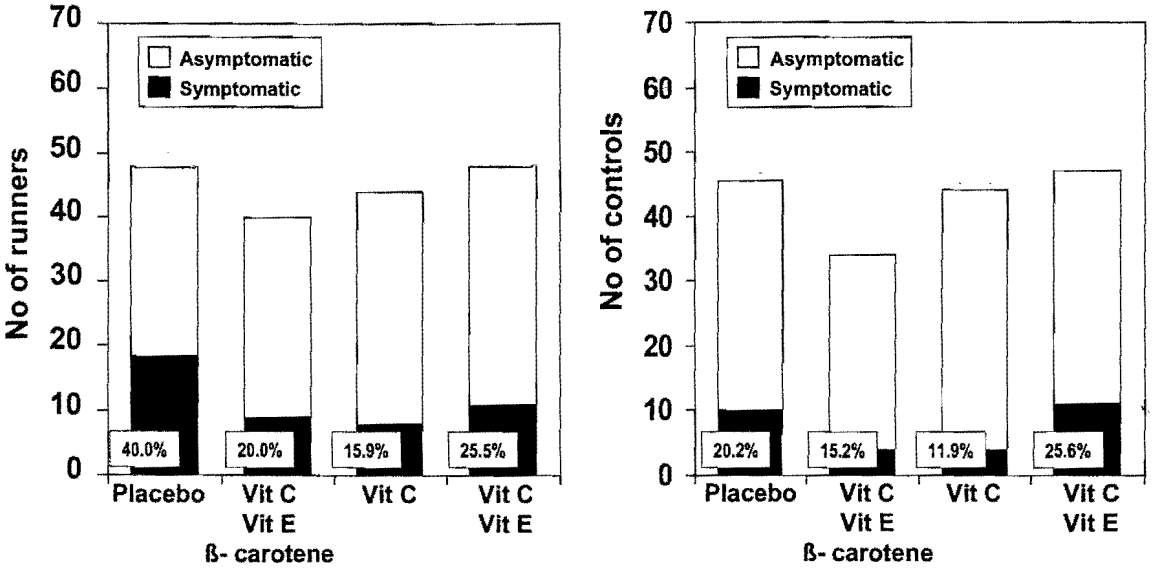

Figure 1.7. Incidence of URTI symptoms in runners (n=178) and controls (n=162) on different anti-oxidant combinations, during the 14 day post-race period. (Peters *et al.*, 1996)

A direct inverse correlation between the combined intake of vitamin C, E and β carotene and incidence of symptoms of URTI did, however, not exist. The relatively lower effect of vitamin E and β carotene supplementation may be attributed to the slow elevation in circulating vitamin E and β carotene levels and the fact that this

supplementation did not extend over long enough a period of time to allow blood levels of vitamin E and β carotene to reach protective levels. Variance in training status and genetic make-up within and between the groups studied, however, also appeared to have an important bearing on the efficacy of anti-oxidant nutrient supplementation in this study.

The major finding of this study, however, supports the notion that a total intake of approximately one gram of vitamin C per day for three weeks prior to the race, does have a protective effect in ultramarathon runners in terms of reducing the URTI risk. This is considerably higher than the daily dosage of 200 mg (for 4 weeks) which has been shown to be associated with accelerated clinical improvement in elderly patients hospitalized with acute respiratory infection (Hunt *et al.*, 1994). The results of this trial also support previous findings of studies examining the effect of vitamin C supplementation on the incidence of URTI in subjects exposed to severe physical stress **(Table 1.1)**.

In 1959 Bessel-Lorck found that the incidence of URTI was 28% lower in 20 vitamin C supplemented children attending a 9-day skiing camp in the Bavarian woods than in 26 non-supplemented children who acted as controls. This was followed by a carefully controlled double blind study conducted on 279 school children in two skiing camps in the Swiss Alps (Ritzel, 1961). Ritzel administered one gram of vitamin C per day to half of these children for a period of a week and reported a decrease in the number of episodes of pharyngitis, laryngitis, tonsillitis and bronchitis in the vitamin C group. In addition to a 45% decrease in the incidence of colds, there was also a 29% decrease in the mean duration of cold episodes and a 61% decrease in the total number of days of illness per person in the group receiving vitamin C. The children in this study were not only exposed to strenuous exercise, but also to the cold, an added environmental stressor (Ritzel, 1961). These findings were supported by those of Sabiston & Radomski (1974) who studied 112 soldiers undergoing military training over a two-week period in the Canadian winter and found less than half the percentage incidence of the common cold in the troops receiving 1g vitamin C per day (11%; n=56) when compared to those on placebo (25%; n=56).

In 1996 Hemilä conducted a meta-analysis of three of the above-mentioned studies of persons exposed to severe physical exertion (Ritzel, 1961; Peters *et al.,* 1993; Sabiston & Radomski, 1974) and reported a pooled ratio of 0.50 of URTI in favour of vitamin C compared to placebo groups during times of heavy stress. Not included in the summed data presented by Hemilä (1996a) were the findings of the study of Pitt and Costrini (1979), which investigated the effect of 2 g/day of vitamin C supplementation vs. placebo during a 2-month military training camp on 674 marine recruits in South Carolina. These findings supported a reduction in the severity of the infections experienced by the military recruits with a substantially lower incidence of pneumonia in the vitamin C group, but did not confirm the findings of a lower incidence of infection observed in previous intervention trials. This study did, however, possess a number of differences in design to the previous studies. As the subjects only received supplementation after two weeks and were followed over a full two-month period, the possibility that an adaptive response, both in terms of physical adaptation and adaptation to the higher intake of vitamin C, may have influenced the effect of the vitamin C supplementation, does need to be taken into consideration (Stanislaw & Klapcinska, 2000). Moreover, as Hemilä correctly observes, the subjects were not subjected to an "acute stress" situation (Hemilä, 1996a).

Another study involving vitamin C supplementation in persons under heavy stress, was conducted by Kimbarowski-Mokrow in the former Soviet Union in 1967. The development of pneumonia was monitored in 226 military recruits who had acquired influenza A infection. In the soldiers who received 300 mg vitamin C supplementation daily (n=114), the incidence of pneumonia was significantly smaller when compared to the incidence in those who received "little vitamin C in food " (n= 112).

Moola (1996) supported the findings of Peters *et al.,* (1992, 1996) that vitamin C supplementation was most effective in reducing the incidence of post-race URTI symptoms in a small group of ultramarathoners, while the reduced effectiveness of a prolonged period of vitamin C supplementation in lowering the incidence of URTI infection symptoms was once again confirmed in the study of Himmelstein *et al.* (1998). In this work, 44 marathon runners and 48 sedentary controls were randomly assigned to a 2-month pre-marathon and 1 month post-marathon regimen of 1000

TABLE 1. 1: VITAMIN C SUPPLEMENTATION STUDIES CONDUCTED ON SUBJECTS SUBJECTED TO HEAVY PHYSICAL STRESS

Authors	Quantity of daily Supplementation	Total daily Vit C intake (mg)	Sample size Active Subjects	Sedentary Controls	Mode of Physical exertion	Duration of Supplementation	% URTI incidence
Bessel-Lorck, 1959	1000mg	not determined	20	-	9-day skiing camp	9 days	17
	non-supplemented		26	-			45
Kimbarowski and	300mg	300mg &	114	-	Soviet military training	not reported	1.8*[a]
Mokrow, 1967	non-supplemented	"little'' in food	112	-			8.9[a]
Ritzel,1961	1000mg	not determined	139	-	7-day skiing camp	1 week	12*
	Placebo		140	-			22
Sabinston & Radomski,	1000mg	not determined	56	-	2 weeks military training	2 weeks-during	11*
1974	Placebo		56	-		training camp	25
Pitt and Costrini, 1979	2000mg	not determined[c]	331	-	8 weeks of military training	8-week study period	90
	Placebo		343	-	in marine recruits		90
Peters *et al*, 1993	Group 1: Vit C: 600mg	1139	43	34	88km run	3 weeks prior to the race	33*[b]
	Group 2: Placebo	494	41	39			68[b]
Peters *et al*., 1996	Group 1: 500mg Vit C& 400IU Vit E	893	40	33	88km run	3 weeks prior to the race	15.9
	Group 2: 600mg Vit C	1004	44	41			20
	Group 3: Vit C: 300mg; Vit E 400IU; Beta Carotene 400IU	665	47	43			25.5
	Group 4: Placebo	585	47	45			40.4
Moola, 1996	Group 1: 600mg Vit C	not determined	11	11	88km run	6 weeks prior to the	30.8
	Group 2: 45mg Beta Carotene		11	11		race	41.7
	Group 3: Placebo		25	19			68
Himmelstein *et al*., 1998	Group1: 1000mg Vit C	1210	41	29	42 km	2 months before	33.3
	Group 2: Placebo	169	30	35		1 month after	43.5

* $p<0.05$ when compared to incidence in unsupplemented groups [a] subjects possessing influenza A who developed pneunonia [b] symtoms lasting ≤ 1 day included in analysis [c] blood vitamin C levels indicated an absence of marginal deficiency in the ontrol group.

mg/ day of vitamin C or placebo. Despite an only small (3.2%), but significant increase in blood vitamin C concentrations in a subsample (n=25) of vitamin C supplemented runners, a 9.9 % difference in the URTI incidence was reported [33.3% vs 42.9 %, among runners receiving vitamin C (n=30) and placebo (n=14) respectively]. Due to the low statistical power of comparisons of these sample sizes, this difference was not statistically significant.

To date, over 60 studies have examined the effect of vitamin C on the common cold. From the data presently available, it would appear that vitamin C supplementation has the greatest effect in children (Hemilä, 1999) and males with low dietary vitamin C intake (Hemilä, 1997) with the greatest benefit from a dosage of ≥ 2g/day (Mink *et al.*, 1998; Hemilä, 1999). While supplementary vitamin C has not been shown to have preventative effects in most normally nourished subjects in Western countries, substantial treatment benefits have been reported in pneumonia and bronchitis patients (Hemilä & Douglas, 1999). There is also strong cumulative evidence which appears to support its possible beneficial effect in reducing the incidence of infection in those exposed to high levels of acute physical stress (Ritzel, 1961; Peters *et al.*, 1993; 1996; Sabiston & Radomski, 1974; Himmelstein *et al.*, 1998). Variables which may affect the efficacy of supplementation in heavily stressed individuals include duration of supplementation period (Stanislaw & Klapcinska, 2000) and intensity and duration of the physical stress (Hemilä, 1996a; Himmelstein *et al.*, 1998). Further work is required to strengthen this evidence and the inclusion of laboratory measurements of circulating vitamin C concentrations, to establish compliance with treatment, is imperative in all further studies.

Chapter Two

Literature Review

2.1 Exercise-induced modulation of the immune system

Exercise has been described as a form of physical stress which is analogous to trauma, tissue damage, burns, spaceflight and sepsis (Pyne *et al.*, 1998) and results in similar changes in the concentration of neuroendocrine hormones to these forms of stress (Brenner, 1998). There is growing evidence that for several hours subsequent to heavy exertion, various components of both innate (natural or non-adaptive) and acquired (adaptive) immunity exhibit significant perturbations. A brief synopsis of these components and their primary functions is given in Table 2.1:

Table 2.1: Primary components of the immune system.

Component	Prevalence in human peripheral blood	Functions
Innate Immunity		
Physical barriers Skin, epithelial cell barriers mucosal secretions	-	First line of defence Contain IgA antibodies
Proteins/polypeptides Incl lysozyme, complement, acute phase proteins, adhesion molecules (e.g. selectins, integrins)	-	-Recruitment of inflammatory cells to site of infection or inflammation, directing leukocyte trafficking -Antibody like activity (binding to cell surface proteins) -Opsonization of pathogens -Stimulation of phagocytosis
Cellular components		
Polymorphonuclear granulocytes	**60-70 % leukocytes**	Phagocytosis, degradation of damaged tissue, cytokine production, presentation of foreign protein to CD4+ cells
Neutrophils	90% granulocytes	
Eosinophils	2.5% granulocytes	
Basophils	2% granulocytes	
Monocytes(CD14),macrophages	**10-15% leukocytes**	Phagocytosis, cytokine production
Natural killer cells (CD16, 56)	5-20% lymphocytes	Cytotoxicity, cytokine production

Acquired Immunity		
Humoral factors Immunoglobulins Soluble messenger molecules (incl. Cytokines, chemokines)		Antigen binding Activation of immune cells & mediation of leukocyte trafficking; acting as chemical messengers between different immune cells Neutralisation/killing pathogens or tumour cells
Immune Cells: Lymphocytes Helper T cells (CD 4+) Cytotoxic /suppressor T cells (CD 8+) B lymphocytes (CD 19- 23)	**20-25% leukocytes** 60-70% T cells 30-40% T cells 5-15% lymphocytes	 Antigen recognition, cytokine production, B cell activation Cytotoxicity, lymphocyte regulation Antibody production, memory of previous infection

Compiled from Janeway & Travers, 1996; McKinnon, 1999; Guyton, 2000.

In support of the apparently consistent findings in epidemiological surveys following long and ultradistance events, laboratory studies have revealed that the following components of the immune system have been shown to exhibit consistent and reproducible change during and following heavy physical exertion:

- **The distribution of leukocyte subsets**: An exercise-induced leukocytosis has been shown to result from increases in the concentrations of circulating adrenal stress hormones (Brenner *et al.,* 1998) and human growth hormone (Kappel *et al.,* 1993). This leukocytosis results from both an elevation of the total lymphocyte count (due to recruitment of natural killer (NK) cells, B cells and T cells into the blood), as well as increased release of neutrophils into the circulation. During the post-exercise recovery, the leukocyte response is, however, biphasic (Hansen *et al.,* 1991; Nieman *et al.,* 1991; 1994). Correlating strongly with the catecholamine curve (Crary *et al.,* 1983), the exercise-induced increment in lymphocyte number has been shown to be reversed after 30 minutes of recovery, dropping down to below pre-exercise levels (post-exercise lymphocytopenia), and together with eosinophil number, remaining low for 3-6 hours (MacArthy & Dale, 1988; Nieman *et al.,* 1989b; 1991; 1994). In contrast, post-exercise neutrophilia (MacArthy & Dale, 1988; Smith *et al.*, 1990; Nieman *et al.,* 1991; Müns, 1993; Pyne *et al.,* 1995) has, in strong correlation with the increment in cortisol levels, been shown to peak at 1.5 hours into recovery and

slowly return to baseline levels between 6 and 20 hours post-exercise (Nieman, 1995; Pedersen *et al.,* 1997). A marked decrease in circulating NK cells (which have been shown to rise 150-300 % immediately following exhaustive exercise and drop to below pre-exercise levels 1-2 hours into recovery), is associated with decreased cytotoxic capacity of the NK cells (MacKinnon, 1989; Berk *et al*, 1990; Pedersen *et al*, 1989; 1990; 1997; Pedersen and Ullum, 1994; Shephard *et al.,* 1994).

- **Systemic markers of inflammation:**

These include **increases** in

- blood granulocytes (primarily neutrophils and eosinophils) and monocyte phagocytic activity (Smith *et al.,* 1990; Müns *et al.,* 1996; Nieman *et al.*, 1996; Suzuki *et al.,* 1996); concentrations of elastase (Gleeson *et al.,* 1998; Robson *et al.,* 1999) and myeloperoxidase (MPO) [Camus *et al.,* 1992; Bury *et al.,* 1995]
- acute phase reactants {including C-reactive protein (CPR), haptoglobin and fibrinogen} [Strachan *et al.*, 1984; Weight *et al.*, 1991; Castell *et al.*,1997; Pizza *et al.,* 1997]
- complement protein levels (Nieman *et al.*, 1989c; Camus *et al.,* 1994; Castell *et al.*, 1997)
- pro-inflammatory cytokines {including tumour necrosis factor-alpha (TNF α), interleukin –1beta (IL-1β)} [Cannon *et al.,* 1997; Ostrowski *et al.,* 1998a; 1999], interleukin-6 (IL-6) [Bury *et al.,* 1996; Brunsgaard *et al.,* 1997; Weinstock *et al.,* 1997; Ostrowski *et al.,* 1998b] and their inhibitors, TNF- receptor and interleukin -1 receptor antagonist (IL-1Ra) [Ostrowski *et al.,* 1999]
- anti-inflammatory cytokines {including interleukin-10 (IL-10)} [Weinstock *et al.*,1997; Nieman *et al.,* 2000; Suzuki *et al.,* 1999; 2000]
- chemokines such as interleukin-8 (IL-8), macrophage/monocyte inhibitory proteins (MIP) -1α and 1 β (Ostrowski *et al.,* 2001)
- mobilisable anti-oxidative vitamins (particularly vitamin C) [Gleeson *et al.,* 1987; Robertson *et al.*, 1991; Maxwell *et al.,* 1993, Liu *et al.,* 1999]

and **decreases** in

- granulocyte oxidative burst following prolonged exercise (Gabriel *et al.*, 1994; 1995; Robson *et al.*, 1999)

Additional components of the immune system which are also consistently suppressed during the post-exercise open window period include

- T and B lymphocyte numbers and function (Liesen *et al.*, 1988; Mars *et al.* 1998; Pedersen *et al.*, 1998a, Nieman *et al.*, 1999)
- salivary IgA output (Mackinnon et al., 1989, 1993a; Mackinnon & Hooper, 1994; Blannin *et al.*, 1998; Fahlman *et al.*, 2001)
- plasma glutamine concentrations (Keast *et al.*, 1995; Castell *et al.*, 1997)

Despite the increasing knowledge of exercise-induced changes in immune function, the clinical significance of these changes is presently not known and the pathophysiological basis of actual increased post-exercise vulnerability/susceptibility to infection among athletes, is currently poorly understood.

2.2 Aetiology of transient post –exercise immunosuppression

It is well accepted that symptoms of infection can be triggered by infective, inflammatory or allergic factors. In 1994 Pederson and Ullum first identified the existence of an "open window" period during the first 6-20 hours following strenuous exertion showing a temporary drop in lymphocyte numbers, NK cell activity, complement and mucosal IgA levels during this time (**Figure 2.1**).

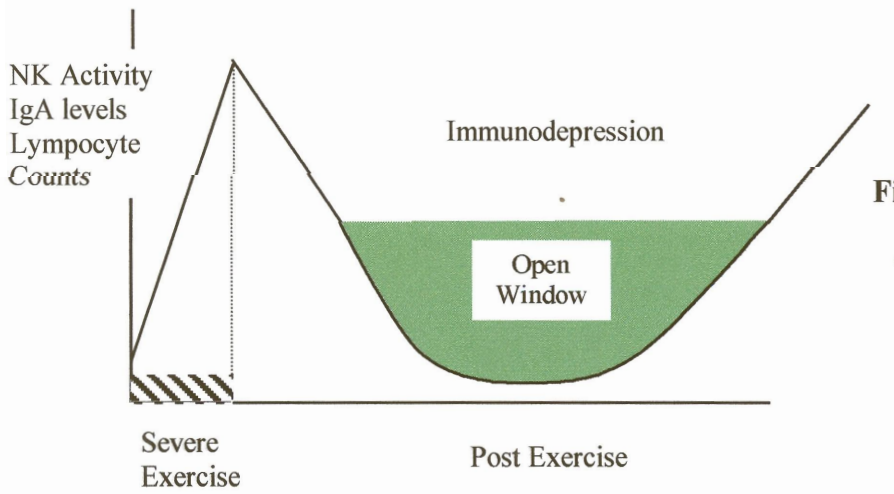

Figure 2.1: The transient post-exercise "open window" period.

This Danish group contend that it is during this transient "open window" period that microbial and viral pathogens can subvert host defenses and the athlete is most vulnerable to developing infections. They subsequently proposed that (i) decreased proliferative response of the lymphocytes (ii) depression of the immune system by corticosteroids produced under physical stress or (iii) harm done to the immune system by oxygen radicals generated during heavy exercise, are possible causes of the transient immunosuppression (Pedersen *et al.,* 1998a).

At present the closest association between markers of reduced immunocompetence and actual infection incidence appears to be between salivary IgA output and the incidence of URTI (Mackinnon & Jenkins, 1993; Pyne & Gleeson, 1998; Gleeson, 2000a, 2000 b). Several studies have shown that salivary IgA concentrations (Tomasi *et al.*, 1982; Mackinnon *et al.,* 1989) and secretion rates (Mackinnon *et al.*, 1993a; Mackinnon & Hooper (1994); Blannin *et al.,* 1998; Fahlman *et al.*, 2001) are depressed following intensive exercise in elite well-trained athletes and have linked these to an increased risk of infection and incidence of URTI in elite athletes (Mackinnon *et al.,* 1993b). Mackinnon & Hooper (1994), examining the secretory IgA response to various exercise conditions, found evidence of a cumulative effect of intense daily exercise on this major effector of resistance against microbial and viral pathogens; well-trained swimmers presented with significantly higher secretory IgA levels than "stale", overtrained swimmers over a 6-month season.

Exercise-induced URTI symptoms cannot, however, be solely attributed to infective origins. A number of excellent reviews have outlined that acute prolonged exercise bouts result in an immunological response which appears to mimic the body's response to inflammation and wound repair (Weight *et al.,* 1991). These include a rise in core body temperature (Cannon & Kluger, 1983), plasma levels of acute phase proteins (Cannon *et al.,* 1991) and cytokines (Pedersen *et al.*, 1998b), accompanied by leukocytosis (Hansen *et al.,* 1991), lymphopenia (Nieman *et al.,* 1995) monocytosis (Nielsen *et al.,* 1996a) and suppressed neutrophil activity (Pyne, 1994).

During prolonged endurance exercise increased ventilatory rates and volumes with actual damage to sensitive mucous membranes in the respiratory tract, and an inflammatory response at the sites of muscle cell damage have been linked to the development of an acute phase reaction (Weight *et al.,* 1991). Shephard & Shek (1996) refer to the "active enmeshment" of the immune system in the muscle tissue repair and inflammation process, and speculate that in this process, protection from respiratory pathogens is compromised.

It has been suggested that the physiologic consequences/metabolic sequelae of sustained exercise are similar, but not analogous, to the acute phase response (Gabriel & Kinderman, 1997). Catecholamines are known to induce a demargination of leukocytes (the increases in leukocyte numbers related mainly to plasma epinephrine concentrations during intense exercise), and as exercise continues, plasma cortisol levels rise inducing an efflux of neutrophils from bone-marrow, and retention of cells in lymphoid tissue. (Brenner *et al.,* 1998). The acute phase response thus manifests with a marked, but transient, neutrophil leukocytosis with concomitant increases in plasma cortisol, creatine kinase (CK), CRP and total protein level. Consistent delayed increases in serum haptoglobin (48 hours post-race) with no change in serum iron level, total iron-binding capacity and serum ferritin concentration have also been reported (Weight *et al.*, 1991). More recent work linking the release of pro-inflammatory cytokines (Ostrowski *et al.,* 1998a, 1989b; 1999) to an acute phase response, have led to the description of a "trauma- like" response to prolonged treadmill running.

Gabriel and Kinderman (1997) have, however, recently emphasized an important distinction between the apparent exercise-induced acute phase protein response and that induced by a bacterial infection. They showed that the leukocytosis following strenuous exercise is associated with impaired granulocyte oxidative burst activity and suppressed defence mechanisms, whereas the leukocytosis present in a bacterial infection is accompanied by priming (sensitising) of neutrophils for increased function and subsequent activation of these cells.

It is well known that indomethacin decreases *in vitro* release of immunosuppressive prostaglandin E_2 from mononuclear cells and restores suppressed post-exercise neutrophil chemiluminescence and NK cell activity (Pedersen *et al.,* 1990). In a work on URTI incidence following participation in the 1996 Two Oceans 56 km Ultramarathon, Schwellnus *et al.* (1997) showed that administration of a topical anti-inflammatory, anti-bacterial spray, Fusafungine, resulted in a lowering of the incidence of URTI in 48 participants when compared to an equal number of runners receiving a placebo during the 9 days following the event.

Gleeson (2000b, 2000c) has recently presented an additional alternative hypothesis to explain the occurrence of URTI when a bacterial or viral cause cannot be identified in the apparently healthy athlete. She proposes that in the case of prior exposure to Epstein-Barr Virus (EBV), exercise-induced suppression of cytotoxic T-lymphocytes may reduce the ability of EBV-specific memory T-cells to maintain control over virus expression and allow EBV shedding into the saliva of asymptomatic healthy carriers. She hypothesises that following exercise-induced disturbance of the cytokine balance and suppression of mucosal antibodies (low levels of salivary IgA), viral reinfection may thus occur in the oral mucosa. These could present with URTI symptoms which would induce a transient inflammatory response and activate the relevant control systems to restore immune functions in previously asymptomatic healthy, elite athletes (Gleeson, 2000b, 2000c).

The most recent state of knowledge thus appears to support the contention that increased infection risk may indeed be caused by the interaction of a combination of current and perhaps prior, pro-infective and pro-inflammatory responses which are modulated by the presence of physical, psychological and environmental stresses placed on the athlete engaging in elite endurance sport. Pyne and Gleeson (1998) further point out that the “transient and modest” nature of the observed changes may be indicative of a "self-modulating immune cell network capable of homeostatic regulation." This perhaps accounts for the rapid post-exercise recovery of most markers of immune response.

2.3 Vitamin C and reduced infection incidence in athletes

In spite of the apparent beneficial effects of vitamin C supplementation on reducing the incidence of post-race URTI, the mechanism by which this is achieved has not been elucidated. The possible involvement of reactive oxidants as mediators of the futile inflammatory response and transient decrease in immune function which accompanies intensive and prolonged exercise was supported by the findings of my three double-blind, placebo-controlled intervention studies (Peters *et al.*, 1992; 1993; 1996) in which statistically significant reductions in the incidence of self-reported symptoms of URTI in the groups supplemented with the anti-oxidative vitamins were observed, with vitamin C being the most effective.

2.3.1 *Properties of vitamin C*

Vitamin C is an organic compound which cannot be synthesized in primates (including humans) and guinea pigs (Burns, 1959). It is thus an essential nutrient in the human diet. The highest concentration of this vitamin is found in the human adrenals, ovaries, brain, pituitary gland, liver, spleen and blood cells (Moser & Bendich, 1991). It has diverse functions in the body which include an essential role in hydroxylation reactions necessary for collagen formation and carnitine synthesis, competitive substrate binding which inhibits the formation of carcinogenic nitrosamines, as well as the facilitation of iron absorption (Burns, 1959; Tolbert, 1985; Glatthaar *et al.*, 1986; Levine, 1986).

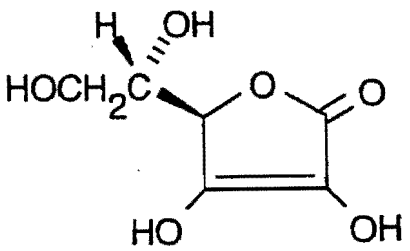

Figure 2.2: Structure of ascorbic acid (AA)

In most animals, the six-carbon ketolactone, L-ascorbate (C_6,H_6,O_8), is synthesised from glucuronic acid or galatonic acid which is derived from D-glucose. Its five membered ring **(Figure 2.2)** includes an endiol substructure with an acidic proton

(pK_a= 4.17) and the compound functions as a reducing agent and co-enzyme in several metabolic pathways.

Perhaps the most widely acknowledged role of ascorbate is that of its function in acting as co-factor for prolyl and lysyl hydroxylases in the biosynthesis of collagen. The hydroxylation steps of collagen biosynthesis are dependent on the enzymes, proline hydroxylase, procollagen-proline 2-oxoglutarate 3-dioxygenase and lysine hydroxylase which are in turn, dependent on AA for maximum activity (Chojkier *et al.,* 1989).

Additional enzymes which are dependent on ascorbate for optimal function include gamma-butyrobetaine and 2-oxoglutararate 4-dioxygenase which are responsible for hydroxylation of carnitine precursors (Otsuka *et al.,* 1999), hydroxypenylpyruvate dioxygenase, which catalyses the hydroxylation and decarboxylation of a tyrosine metabolite (Levine, 1986; Moser, 1992) dopamine β-monooxygenase, an enzyme active in norepinephrine biosynthesis (Dhariwal *et al.,* 1989; Moser, 1992) as well as several enzymes involved in hydroxylation reactions of the cortisol synthesis pathways (detailed on page 30).

2.3.2 *Anti-oxidant actions of vitamin C:*

In addition to its importance in many enzymatic reactions, an important explanation of the protective action of vitamin C during exercise may relate to its ability to directly deactivate/ neutralise the free radicals and other reactive oxidants which are produced during prolonged exercise and oxidative metabolism (Zembron-Lacvny & Szyszka, 2000). AA has been demonstrated to be the most efficient water-soluble anti-oxidant of blood and tissue fluids (Frei *et al.*, 1989). It can act directly with aqueous oxygen- derived reactive species by donating one or two electrons to metal ions, or within redox systems (e.g. hydroxylation reactions). The oxidised form, dehydro-ascorbic acid (DHAA), is immediately converted back to the reduced form. Its anti-oxidant function can also be indirect, by restoring the anti-oxidant potential of the fat-soluble vitamin E when donating an ascorbate electron to the tocopherol radical (Packer, 1986; Moser, 1992) **[Figure 2.3].**

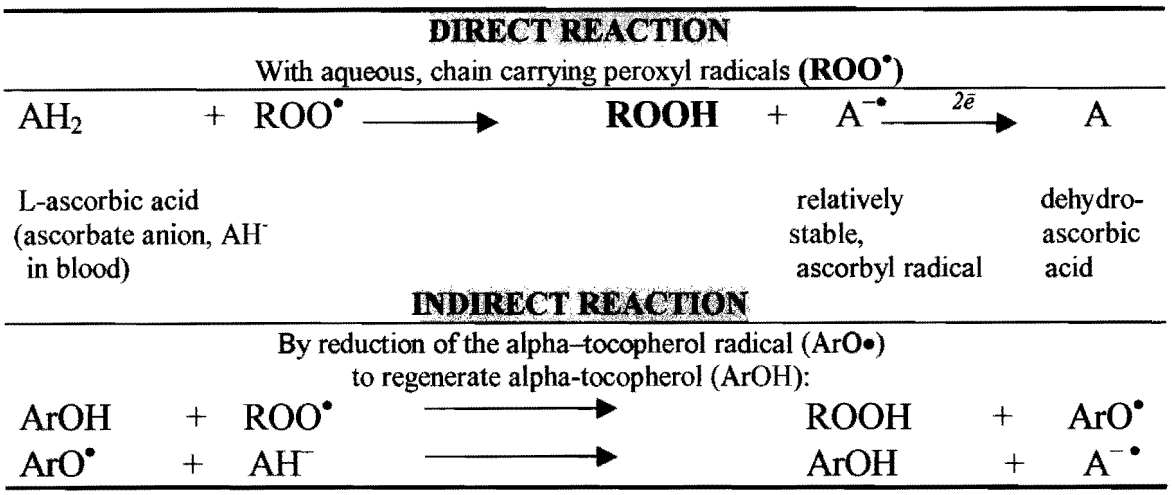

Figure 2.3: The anti-oxidant effect of vitamin C: direct and indirect processes.

It has also been proposed that high AA levels in neutrophils (Moser & Weber, 1984) protect against self-destructive autooxidation (Peters-Futre, 1997). AA is known to scavenge and neutralize effectively $O_2\bullet^-$, •OH, singlet oxygen and HOCl generated during the process of neutrophil activation (Anderson, 1981; 1991, 1995; Bendich *et al,* 1986; Smith *et al.,* 1990b; Peters-Futre, 1997; Ohno *et al.*, 1998; König *et al.,* 2001) **[Figure 2.4].** Release of AA into the extracellular fluid by neutrophils is therefore thought to protect these and other cells from free radical damage (Moser & Weber, 1984; Bendich *et al.,* 1986; Wasko *et al.*, 1989).

Chronic activation of phagocytes resulting from autoimmune disease and infection has been linked to a depletion in total and spleen ascorbate stores (Washko, 1989), as well as to an increase in the oxidised fraction of vitamin C, DHAA (Padh, 1990). Despite evidence of a rise in plasma ascorbate concentrations during prolonged exercise (Fishbaine & Butterfield, 1984; Gleeson *et al.,* 1987; Duthie, 1990; Koz *et al.*, 1992) and an adaptive up-regulation of the anti-oxidant defence system following sustained periods of endurance training (Duthie, 1997; Himmelstein *et al.*, 1998; Brites *et al.*, 1999), prolonged exercise has been associated with elevations in non-mitochondrial generation of reactive oxygen species (ROS) [Davies *et al.*, 1987; König *et al.*, 2001] and a rise in the ratio of oxidised to reduced vitamin C (Siegel & Leibovitz, 1982). Phagocytes and lymphocytes contain greater than 10-fold the

concentration of L-ascorbic acid in blood plasma (Wasko *et al.,* 1989). This, together with the high concentrations of Vitamin C stored in the adrenal and pituitary glands (which can reach about 150 times the concentrations in plasma), suggest the possibility of functional roles of this vitamin in the cells of the immune system during exercise (Moser, 1992; Redman *et al.*, 1995). Animal studies show that AA is involved in regulating a number of neutrophil functions, including chemotactic responses, phagocytosis, hexose monophosphate shunt activity and MPO function (Anderson, 1995). Suppression of phagocytic activity been shown to occur only when AA has been depleted at sites of vigorous phagocyte activity (Van Antwerpen *et al.,* 1991). This suppression has, in turn, been reversed following administration of the vitamin (Anderson *et al.,* 1989; Frei *et al.,* 1989; Wasko *et al.,* 1989).

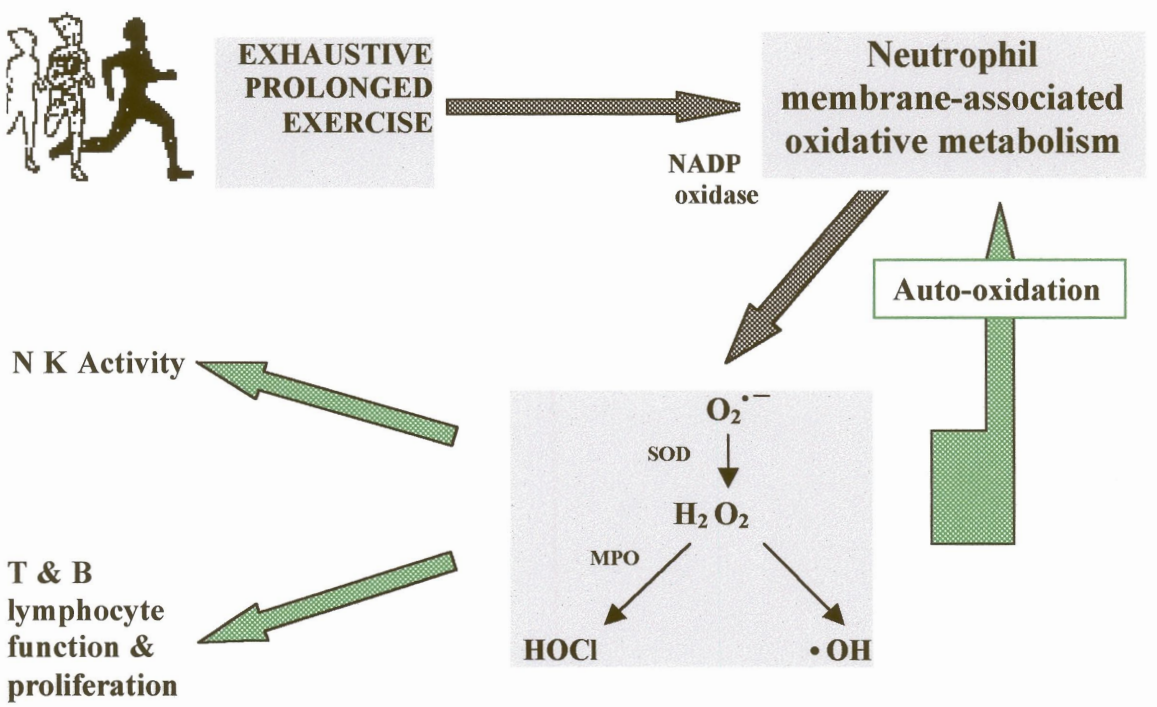

Figure 2.4: Exhaustive, prolonged exercise enhances neutrophil membrane-associated oxidative metabolism. The reactive oxidants produced, in turn, damage the neutrophils, suppressing further oxidative activity in these cells, and cause oxidative inhibition of T and B lymphocyte function and proliferation and NK activity. Adapted from Peters, 1997. ⟹ positive ⟹ negative.

Findings of *in vivo* and *in vitro* measurements in normal and immunocompromised human volunteers and in experimental animals appear to indicate that the concentration of vitamin C is a significant factor in neutralizing the escaped oxidants which accumulate when pro-oxidative events overwhelm available anti-oxidant protection. Anderson & Theron (1979) reported that *in vitro* incubation of human neutrophils with calcium or sodium ascorbate causes stimulation of neutrophil motility and migration to chemoattractants. Neutrophil motility was further investigated *in vivo* following the ingestion of increasing weekly doses (1,2,3 g daily) of sodium ascorbate by normal adult volunteers. Significant stimulation of neutrophil motility was observed following ingestion of 2 g or 3 g daily and a mechanism involving inhibition of MPO-mediated iodination was proposed.

The stimulatory effects of ascorbate on human neutrophil motility *in vivo* and *in vitro* have been associated with inhibition of the autooxidative effect of the MPO/ H_2O_2/haldide system (Anderson, 1981). In a study in which the effect of a single intravenous injection of 1g of ascorbate was monitored, the immunostimulatory and peroxidase inhibitory activity was further related to serum ascorbate level. Anderson *et al.* (1989) identified that a primary biological function of ascorbate in host defence was to neutralize granulocyte-derived HOCl, sustaining the functions of phagocytes by protecting these cells from auto-oxidation by products of their own oxidative metabolism (Anderson & Lukey, 1987).

Evidence is thus accumulating in favour of local vitamin C concentrations at sites of vigorous phagocyte activity being a primary determinant of the magnitude and/or duration of protective immune responses. Supporting the reactive oxidant scavenging properties of vitamin C *in vivo*, an inhibitory effect of Vitamins C and E on the production of phagocyte-derived extracellular ROS has been reported by Herbacynska-Cedro *et al.* (1994). The effect of oral supplementation of vitamins C and E (600 mg of AA and alpha-tocopherol acetate per day for 14 days) to 13 healthy volunteers on leukocyte production of ROS was estimated by lucigenin amplified chemiluminescence in isolated leukocytes that had been stimulated with arachidonic acid. These findings of an inhibition of phagocytic ROS production

following vitamin C supplementation were further confirmed in patients with myocardial infarction (Herbacynska-Cedro *et al.,* 1995) and coronary heart disease in the setting of significant decreases of serum levels of lipid peroxides (de la Fuente *et al.,* 1998), lending support to the theory that anti-oxidant vitamins may provide vasoprotection.

Recent studies have also provided evidence of the importance of AA in mononuclear cells. Schwager and Schulze (1997a; 1997b; 1998), investigating the effect of AA supplementation on lymphocyte function in young pigs who were unable to synthesise ascorbate endogenously, found that increasing extracellular ascorbate concentrations were not only associated with reduced activation of T and B lymphocyte in response to both pokeweed mitogen (PWM) and LPS, and reduced interleukin-2 (IL-2) production by activated lymphocytes (Schwager & Schulze, 1997a), but also decreased production of reactive oxygen intermediates (ROI) by polymorphonuclear leukocytes after supplementation with 5 and 50 mg AA per kg per day (Schwager & Schulze, 1997b). The findings of subsequent work appear to indicate that ascorbate reduces ROI levels via its effect on IL-2R expression and suggests an inverse relationship between cellular levels of ascorbate and the activity of different genes in lymphoid cells (Schwager & Schulze, 1998).

Cooke *et al.* (1998) examined the effects of supplementation with 500 mg vitamin C per day on *in vivo* levels of oxidative DNA damage. Levels of 8-oxo-2-deoxyguanosine (8-oxodG) in molecular cell DNA, serum and urine of human subjects, were significantly decreased which correlated strongly with increases in plasma vitamin C concentration. This is the first evidence in humans which suggests a positive, anti-oxidant role for vitamin C in the regulation of DNA repair enzymes.

The most recent work of Dietrich *et al.* (2002) also supports the numerous *in vitro* findings that vitamin C ameliorates lipid peroxidation which result from exposure to free radicals (Frei, 1994). In a randomised double blind placebo-controlled trial performed on 126 cigarette smokers, 2 months of daily supplementation with 500 mg of vitamin C decreased plasma F2-isoprostane levels in subjects with a high body

mass index by 28.8 pmol/litre when compared with the placebo group (p=0.001). This *in vitro* work supports a protective function of vitamin C in reducing the oxidative damage caused to lipids in cigarette smokers, but what of the effect of vitamin C on the production of free radicals and oxidative damage induced by participation in prolonged exercise (Davies *et al.*, 1987)?

Although two recent works conducted on small sample sizes (Nieman *et al.*,1997; Krause *et al.*, 2001), did fail to show that vitamin C supplementation significantly affected post-exercise neutrophil function (including on neutrophil phagocytosis, bacteriocidal capacity and oxidative burst), several recent studies (Duthie *et al.*,1996; Alessio *et al.*, 1997; Sanchez-Quesada *et al.*, 1998; Vasankari *et al.*, 1998; Ashton *et al.*, 1999; Schröder *et al.*, 2000) have provided evidence of reduced post-exercise oxidative stress following an acute period of vitamin C supplementation.

The effect of Vitamin C supplementation (1g/day) for 7 days and 2 weeks on biomarkers of pro-oxidative plasma thiobarbituric acid reacting substances (TBARS) and anti-oxidative activity oxygen radical absorbance capacity (ORAC) was determined using the TBARS: ORAC ratio to represent oxidative stress (Alessio *et al.*, 1997). This ratio was highest (32%) following 30 min of running exercise when a placebo was given and only rose by 5.8% after one day of vitamin C supplementation as opposed to 25.8% after 2 weeks of supplementation. As the increases in oxidative stress ratios, however, did not reach statistical significance, this study appeared to only support a mild tendency of biomarkers of oxidative stress to tilt the oxidative stress balance towards antioxidant activity after vitamin C supplementation. Of interest, is the apparently more marked effect after an acute period of supplementation than the more prolonged two-weeks of supplementation which appear to support previously reported findings of Duthie *et al.* (1996) and Schröder *et al.*(2000), but require further confirmation.

Ashton *et al.* (1999) have found that acute supplementation with 1000 mg of L-ascorbate in 10 subjects 2 hr before an incremental exercise test to exhaustion, resulted in a significantly lower post-exercise lipid hydroperoxide, malondialdehyde and

electron spin resonance signal intensity. Vasankari *et al.* (1998), studying the effects of supplementation of 2 g vitamin C per day versus placebo on 9 athletes on oxidative stress following a longer 10.5 km maximal run, showed that serum diene conjugation concentration decreased by 11% following the vitamin C trial, but not in the placebo ($p=0.03$), while Sanchez-Quesada *et al.* (1998) confirmed evidence of reduced oxidative stress following a 4-hour athletic race in subjects receiving oral supplementation of 1g of AA per day. In this study, exercise-induced increases in susceptibility of low density lipoprotein (LDL) to oxidation and proportion of LDL(-) were inhibited in the group receiving AA. Schröder *et al.*(2000) provided further evidence of the anti-oxidant actions of vitamin C in exercising individuals with their finding of a 15.3% reduction in the lipid peroxide/total anti-oxidant status ratio (measured by chromogenic method) in professional basketball players following 32 days of supplementation with a three-compound anti-oxidant supplement containing 600 mg α tocopherol, 1000 mg vitamin C and 32 mg β-carotene.

Taken together, the current evidence in favour of protective anti-oxidant effect following Vitamin C supplementation in acutely stressed individuals, is convincing.

2.3.2 *Pro-oxidative potential of vitamin C*

The potentially harmful effects on health of high intakes of AA have been the subject of much debate. In addressing the question of ascorbate toxicity which may be associated with large doses of supplemental intake of vitamin C, most studies have focused primarily on potential increases in oxalate formation, decreased uric acid excretion, impairment of vitamin B status and iron overload. Since kinetic studies using isotopes have proved that the metabolic turnover of vitamin C is limited (with the maximum reaching about 40 mg/day), large doses of vitamin C should not result in increased oxalate and subsequent kidney stone formation (Schmidt *et al.,* 1981). This was shown by Hagler and Herman (1973), who found that daily intakes of 10 g only increased urinary oxalate excretion by 15 to 37 mg per day, which is no more than that resulting from "normal" dietary intake of food, and has subsequently been confirmed by Schmidt *et al.* (1981), Gerster (1997) and Curhan *et al.* (1999).

Physiologists have reasoned that an increased load of AA in the proximal tubule, may, due to competitive inhibition, decrease uric acid reabsorption and lead to ascorbic-acid induced uricosuria (Guyton, 2000). This has not been confirmed by the literature. Relatively small increases in urate excretion at high, non-physiological plasma ascorbate levels, have led to the suggestion that urate may possess a preferential affinity for this transport mechanism or an additional secretory transport system not shared with ascorbate (Rivers, 1987).

While research evidence has consistently demonstrated that vitamin B_{12} in food or the body is not destroyed by AA, reports of possible enhancement of pro-oxidative activity following supplementation with vitamin C do, however, exist (Shilotri & Bhat, 1977; Herbert, 1993).

The theory that megadoses of vitamin C have a pro-oxidant effect was first related to its reaction with iron; that in the presence of iron, AA converts iron stores to catalytic iron which possesses strong pro-oxidant effects (Salonen *et al.*, 1992). An almost optimal iron absorption is obtained with 25-50 mg AA per meal, refuting the possibility of a linear enhancement of iron absorption by the vitamin in healthy, iron-replete individuals (Hallberg, 1985; Bendich & Cohen, 1990). The concern was, however, that in the case of persons born with a gene for increased iron-absorption, high vitamin C intake, which is known to increase absorption of dietary iron (Hallberg, 1985), can cause iron overload and the release of large amounts of catalytic iron from their body stores (Gerster, 1999). This is, however, only applicable if serum ferritin levels are in excess of 120µg/l (Salonen *et al.*, 1992). As these concentrations have rarely been described in elite athletes and are not considered physiological, the possibility of an iron-associated pro-oxidant effect of vitamin C in athletes is unlikely.

Shilotri & Bhat (1977) reported that supplementing adult human volunteers with 200 mg as well as 2000 mg vitamin C per day stimulated hexose monophosphate shunt activity of resting neutrophils. Although bactericidal killing activity was not affected by the moderate dose of vitamin C supplementation, administration of a megadose resulted in a decrement of bacterial killing of the leukocytes. As the megadose of vitamin C

administered was not accompanied by an increase in plasma cortisol, and circulating levels of cyclic nucleotides were not measured, these authors were unable to clarify possible mechanisms with certainty.

A recent debate revolved around the findings of Podmore *et al.*(1998) that administration of 500 mg of Vitamin C to 30 healthy volunteers for 6 weeks resulted in a decrease in 8-oxo-7,8 dihydroguanosine, and an increase in 8-oxo-7,8 - dihydroadenosine in lymphocyte DNA. A major criticism of this study is that intracellular vitamin C concentrations were not measured in the lymphocytes and that increasing the extracellular plasma concentration of ascorbate above 50 µM should not have affected the already saturated intracellular concentration of lymphocytes further (Levine *et al.*, 1998). As the study was not placebo-controlled or double blinded, further well-designed trials are needed to confirm this finding.

Nevertheless, caution is merited and careful consideration needs to be given to possible mechanisms by which vitamin C may exert pro-oxidative effects *in vivo*. On the one hand, Anderson has, based on *in vitro* observations, proposed that vitamin C possesses three properties which might contribute to pro-oxidative activity *in vivo*. Firstly, the vitamin does not scavenge H_2O_2, a cell-permeable, reactive oxidant (Anderson & Lukey, 1987). Secondly, and somewhat paradoxically, vitamin C, by acting as a scavenger of HOCl, prevents auto-oxidative inactivation of NADPH-oxidase, resulting in increased production of H_2O_2 by activated phagocytes (Anderson & Lukey, 1987). Thirdly, vitamin C, probably by complexing with the critical heme group of catalase, inhibits the H_2O_2, neutralizing activity of this enzyme (Orr, 1967; Poulsen *et al.*, 1998). If operative *in vivo*, these pro-oxidative activities of vitamin C may predispose to H_2O_2 - mediated tissue damage and genotoxicity as a result of both increased production and reactivity of this reactive oxidant.

At this stage there is, however, not enough concrete evidence to support the possibility of a dualistic, differential response to vitamin C supplementation. To quote Poulsen *et al.* (1998) "it is too soon to say whether supplemental doses of vitamin C exert pro-oxidant or mutagenic effects." The rare incidences of conflicting evidence do,

however, justify the need for further research in order to confirm the correctness of the present assumption that megadoses of vitamin C have beneficial anti-oxidant effects to exercising individuals and clarify the reasons for the occasional discrepant findings.

2.3.4 *Suppressed production of cortisol by adrenocortical cells from the adrenal gland as an alternative mechanism of Vitamin C mediated immunostimulation*

In the 1940s vitamin C was first labelled as an "anti-stress" vitamin. This claim was subsequently refuted due to the absence of adequate research data to substantiate it. It has, however, recently been shown that vitamin C may antagonize the immunosuppressive effects of corticosteroids by interfering not only with their synthesis and/or release (Pardue & Thaxton, 1984a), but also with their interactions with target cells (Schwager & Schulze, 1998; Bowie & O'Neill, 2000; Horton *et al.*, 2001).

Numerous reports have indicated that the synthesis and/or release of the major, endogenous immunosuppressive glucocorticoid, cortisol, is regulated by vitamin C (Pardue & Thaxton, 1984a; Goralczyk *et al.*, 1992; Moser, 1992). While vitamin C infusion has been found to enhance adrenal corticoid production in the laboratory of Kodama *et al.* (1994; 1996), the evidence is accumulating in favour of lowered serum cortisol concentrations with and without increases in ACTH when vitamin C status is enhanced. Laboratory work has showed that increasing ascorbate levels in adrenal cortices results in an inhibition of steroidogenesis (Kitabchi, 1967; Sulimovici & Boyd, 1968; Siegel, 1971) and an association between increased dietary intake of vitamin C and a reduction in stress-related increases in circulating corticosteroids has also been described in poultry (Pardue et al. 1985a; Satterlee *et al.*, 1989, 1994; Jones *et al.*, 1999), guinea pigs (Odumosu, 1982; Enwonuwo *et al.*, 1995), rats (Campbell, pers commun), adult patients undergoing surgery (Nathan *et al,* 1991) and elderly women (de la Fuente *et al.*,1998]. Liakakos *et al.*(1975) have shown that AA administration to children reduced mean plasma cortisol values following administration of ACTH. Sumbaev & Iasinskaia (1997) confirmed these

findings by showing that AA not only regulates corticosteroid production, but also activates the hydroxylation of adenyl purines and uric acid in both animals and humans. While Farr *et al.* (1997) provided evidence of reduced weight-loss and less dehydration in AA supplemented broiler chicks exposed to slaughter stress, McKee *et al.*(1997) reported increased weight gain with increased plasma triglyceride concentrations following heat-induced stress in AA supplemented broiler chicks and Campbell (pers commun) evidenced less weight loss together with reductions in thymus involution and adrenal hypertrophy in stressed rats fed 200 mg (the equivalent of a few thousand milligrams in humans) vitamin C per day. It is of interest that each of these sets of findings following AA supplementation appears to be compatible with lowered stress-induced corticosteroid production.

One possible cause of AA- induced reduction in circulating corticosteroid levels may be inhibition of the functional capacity of several enzymes involved in adrenal corticosteroid biosynthesis by vitamin C (Pardue & Thaxton, 1984a; Satterlee *et al.*, 1989). The specific cortical enzymes involved in steroid synthesis are depicted in **Figure 2.5.**

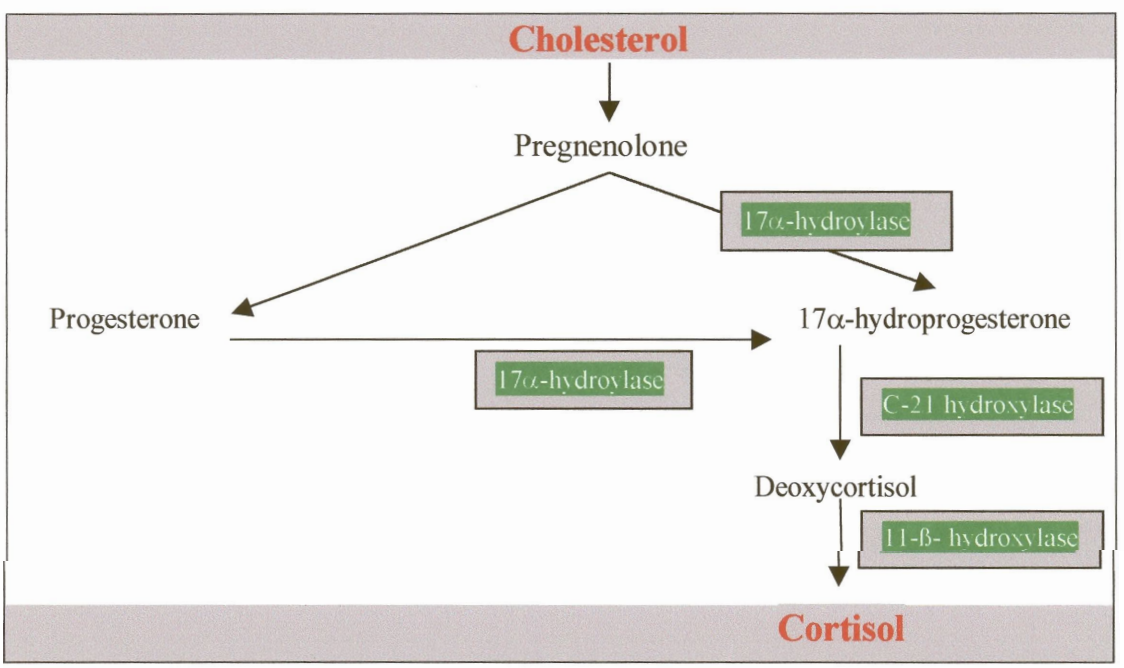

Figure 2.5 Cortical enzymes which regulate adrenal biosynthesis of cortisol (Lindzey & Korach , 1997).

Evidence is available that high adrenal AA levels reduce plasma corticosteroid concentrations due to the ability of the vitamin to (i) inhibit the enzymatic side chain cleavage system that converts cholesterol to pregnenolone (Sulimovici & Boyd, 1968; Shimizu, 1967) and (ii) inhibit C-21 hydroxylase and 11-ß- hydroxylase in the steroidgenesis pathways (Hayono *et al.,* 1956; Cooper and Rosenthal, 1962; Kitabchi, 1967) [**Figure 2. 5**]. Administration of ACTH has, in turn, been reported to inhibit AA transport into the adrenal cortex of the rat and this inhibition has been correlated with a concomitant increase in steroidgenesis (De Nicola *et al.,* 1968).

The immunosuppressive effects of corticosteroids, so-called steroid mediated immunosuppression (SMI), have been reported in numerous species. Following prolonged exercise elevated circulating cortisol levels have been related to decreases in peripheral blood lymphocyte number and proliferation, decreased NK cell cytotoxicity and suppression of neutrophil function (Farrell *et al.*, 1983). A reduction in post-exercise circulating cortisol levels resulting from enhancement of circulating ascorbate levels, would thus have important implications in terms of reducing transient post-exercise SMI. This is also a potential mechanism by which vitamin C increases the proliferative responses of T-lymphocytes *in vitro* (Siegel & Morton, 1977; Anderson *et al.,* 1989; Campbell *et al.*, 1999) and in human subjects (Anderson *et al.*, 1980; de la Fuente, 1998).

2.3.5 *Protection from steroid insult*

Not only has vitamin C supplementation been linked to decreased synthesis of cortisol, but in experiments conducted on broiler chickens, it was found that supplemental AA provided in the diet significantly protected cells from the cytotoxic effects of adrenal steroids and reduced the immunosuppressive effects of elevated circulating cortisol concentrations. These included reduced heterophil/lymphocte ratios (Satterlee *et al.,* 1989), increased agglutinin production (Pardue & Thaxton, 1984b) as well as ameliorated heat-mediated immunosuppression in chicks challenged with sheep erythrocytes (Pardue *et al.*, 1985a) and heat-associated growth inhibition and mortality (Pardue *et al.*, 1985b). Although the previously described

anti-oxidant properties of AA may protect cell membranes from steroid-induced injury, Moffat *et al.*(1972) suggest a possible protective mechanism related to cyclic AMP (cAMP) production within lymphoidal tissue which may account for the findings of Pardue *et al.* (1984b; 1985a; 1985 b). This possibility also requires further verification.

2.3.6 *Reduced exercise-induced mobilisation of vitamin C stores*:

Mobilization of vitamin C from the adrenals has been shown to be a component of an adaptive response to regular and repeated exposure to oxidative stress. This has been confirmed in "fit" runners (Bergholm *et al.*, 1999), runners in a high-training group (Robertson *et al.*, 1991), trained marathon (Gleeson *et al.*, 1987) and ultramarathon runners (Peters *et al.*, 2001a, 2001b - which form a component of this thesis) and in a diverse range of 44 athletes (Rokitzki *et al.*, 1994a), all of whom were found to present with elevated circulating ascorbate levels when at rest viz. well above the 42.4 µmol/l which Brubacher *et al.* (2000) report as the 50th percentile of the plasma concentration for a daily vitamin C intake of 60 mg/day. In addition, Himmelstein *et al.*(1998) found mean plasma vitamin C concentrations were 29.2 % higher in 44 registered participants in the 1994 Duke City Marathon as opposed to those in 48 sedentary controls (80.3 ± 2.99 vs 56.8 ± 4.86 µmol/l), while Brites *et al.*(1999) found that the total plasma anti-oxidant capacity was 25% higher in a group of soccer players engaged in regular training than in matched, sedentary controls. Higher plasma AA, as well as uric acid, α tocopherol and superoxide dismutase were concluded to reflect a compensatory adaptation to high levels of oxidative stress in sportsmen by these researchers. The work of Schröder *et al.* (2000) did, however, not confirm this in 16 professional basketball players ($\bar{X}$ vitamin C, 50.8 ± 22.6µmol/l), but this is most likely due to the relatively small component of aerobic (oxygen-dependent) training completed by these sportsmen.

In 1960 Lipscomb & Nelson found that AA concentration was increased in venous blood from the adrenals preceding onset of glucocorticoid release. Subsequent laboratory studies have shown that the stimulation of cultured porcine adrenocortical

cells by adrenocorticotrophic hormone (ACTH) in the presence of AA results in the production of cortisol with concomitant release of AA (Moser, 1992) which is dose-dependent (Goralczyk *et al.*, 1992). It is therefore possible that mobilisation of vitamin C from the adrenals in response to acute exercise bouts, may be coupled to increased production of cortisol during prolonged exercise. It is well accepted that adrenal cortisol release is a necessity to counter inflammation-mediated tissue damage which is known to result from exercise in athletes (Clarkson *et al.*, 1992) and the majority of studies which have assessed plasma, serum and lymphocyte AA concentrations following acute exercise bouts, have reported an exercise-induced rise in both circulating cortisol and AA concentration (Gleeson & Maughan, 1986; Gleeson *et al.*, 1987; Duthie *et al.*, 1990; Liu *et al.*, 1999, Viguie *et al.*, 1993). However, in recent work on vitamin C supplemented ultramarathoners (Peters *et al,* 2001a, 2001b, which forms a component of the work presented in this thesis), it was found that additional intake of this vitamin (≥1500mg/d) results in an attenuation of both exercise-induced mobilisation of vitamin C and cortisol, adding support to the possibility that exercise-induced adrenal release of cortisol and AA is coupled.

2.3.7 *Immune neuro-endocrine interactions*

Interactions between the immune and endocrine systems are known to play an important role in maintenance of immune homeostasis, particularly in view of the immunosuppressive properties of the glucocorticosteroids. Glucocorticoid release is stimulated by polypeptides known as cytokines, many of which are synthesised and released in the adrenal gland and modulate its secretory activities, as well as help control and mediate interactions among cells involved in immune responses.

2.3.7.1 Cytokine-mediated activation of glucocorticoid release

The onset of inflammation following muscle cell damage is brought about by the release of the "early" or "alarm" pro-inflammatory cytokines, tumour necrosis factor alpha (TNFα) and interleukin-1β (IL-1β), from tissue macrophages, smooth muscle cells and fibroblasts. These act on fibroblasts and endothelial cells and many other

cell types to induce an inflammatory cascade resulting in the production of interleukin-6 (IL-6) and interleukin-8 (IL-8), which act synergistically with TNFα and IL-1β to influence the interaction between the hypothalamic–pituitary-adrenocortical (HPA) axis and the immune system (Marx *et al.*, 1998). These pro-inflammatory cytokines are released sequentially and have been shown to stimulate the release of glucocorticoid hormones, which may represent a homeostatic, counter inflammatory mechanism to dampen excessive immune responses. A number of recent investigations suggest that adrenocortical and adrenomedullary cells have specific receptors for TNFα, IL1β, IL-2 and IL-6 and that these cytokines are also synthesised in adrenocortical and chromaffin cells. *Escherichia coli* LPS is the most potent stimulator of cytokine biosynthesis, not only in leukocytes and macrophages, but also in adrenal cells (Marx *et al.*, 1998). TNFα, IL-2 and IL-6 directly stimulate glucocorticoid production by the cells of the *zona reticularis*, whereas IL-1β has an analogous effect, stimulating catecholamine release by chromaffin cells or activation of the corticotropin-releasing hormone (CRH)/ACTH system (Nussdorfer & Mazzocchi, 1998).

Buckingham *et al* (1994) have reported that oral or peripheral administration of IL-1ß, IL-1α, IL-6 and IL8 to adult male rats, produce increases in serum cortisol concentration and the release of CRF-41 and arginine vasopressin (AVP) from the hypothalamus, while none of these cytokines directly influenced the release of ACTH from pituitary tissue *in vitro.* Päth *et al.*(1997) confirmed these findings showing that IL-6 receptors predominate in the *zona reticularis* and inner zone and that adrenal steroidgenesis could be stimulated by IL-6 in the absence of ACTH, confirming the importance of this pleiotropic cytokine in initiating a suppression of inflammatory activity.

Presently, it can be concluded that IL-6, which is produced in larger amounts than any other cytokine following exercise, may act on the HPA at three levels (**Figure 2.4**):

1. the hypothalamus by stimulating the secretion of CRH
2. pituitary corticotropes by eliciting adrenocorticotropin hormone (ACTH) release

3. the adrenal gland by enhancing steroid-hormone secretion by adrenocortical cells through direct or indirect paracrine mechanisms.

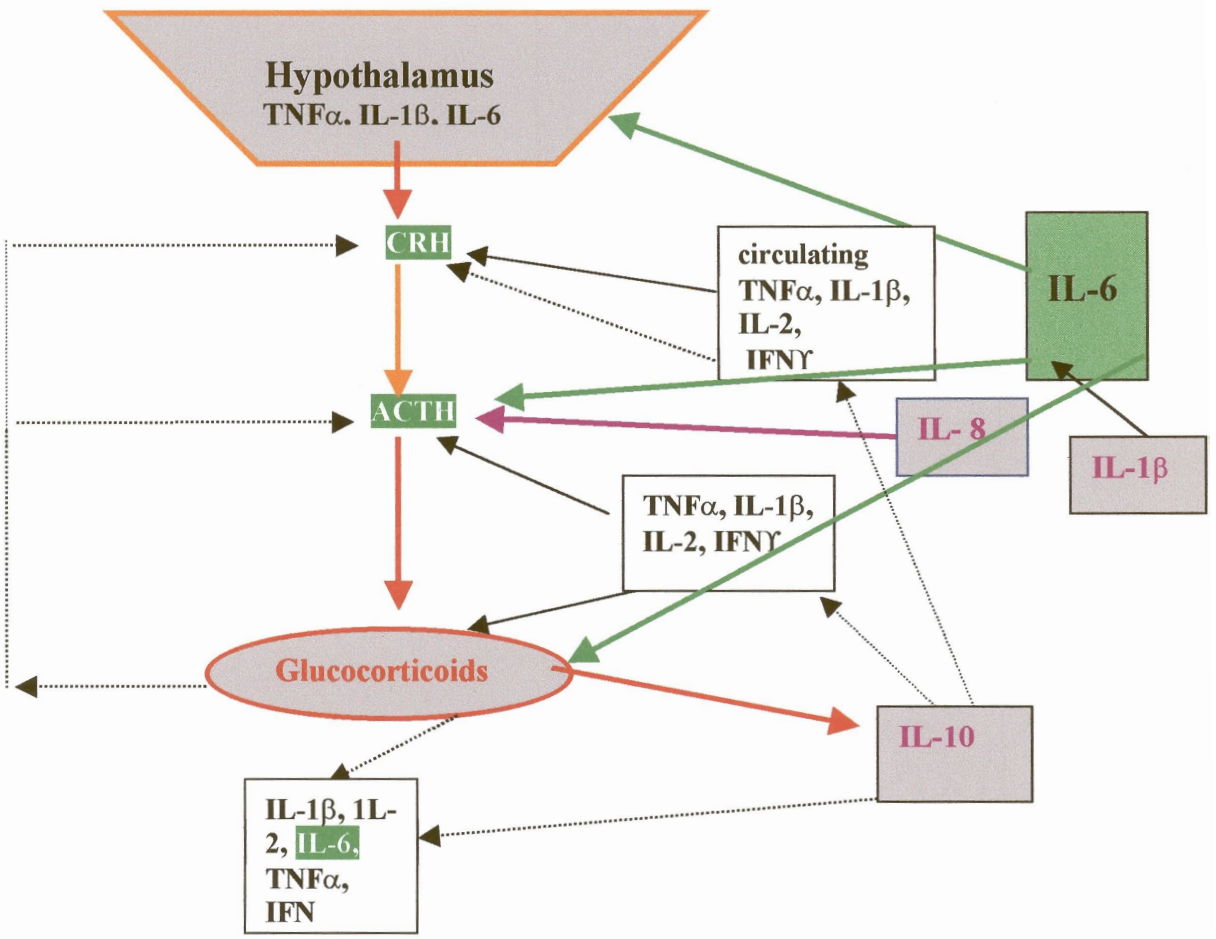

Figure 2.4: The influence of selected cytokines on the hypothalamic- pituitary – adrenal axis ⟶ negative feedback; ⟶ positive feedback (De Waal *et al.*, 1991; Mandrup-Poulsen *et al.*, 1995; Skinkai *et al.*, 1996)

The immunosuppressive activities of corticosteroids affect a wide range of immune and inflammatory cells and their mediators, and are achieved by activation of synthesis of various immunomodulatory polypeptides through the interaction of glucorticoid/ glucocorticoid receptor complexes with glucocorticoid response elements on the promoter regions of steroid responsive genes, as well as by inhibition of the activation of the cytosolic nuclear transcription factors, kappaB (NF*k*B) and activator protein –1 (AP-1), which induce the production of a series of

pro-inflammatory polypeptides (Barnes & Adcock, 1993; Barnes & Karin, 1997; Rahman & MacNee, 1998).

Activation of transcription factor, NF*k*B, a mediator of altered gene expression during inflammation, and implicated in viral infection, has been reported to be inhibited by AA (Schwager & Schulze, 1998; Bowie & O'Neill, 2000; Horton *et al.*, 2001). Schwager & Schulze (1998a) suggest that ascorbate affects immune homeostasis via reactive oxygen intermediate (ROI)–dependent expression of interleukin genes due to sensitivity of NF*k*B to ROIs. Bowie & O'Neill (2000), however, showed that inhibition of NF*k*B by AA was not simply an anti-oxidant effect as redox-insensitive pathways to NF*k*B were also "blocked." Inhibition of TNF-driven I-kappa B kinase (IKK), which inhibits degradation and phosphorylation of I-kappa alpha, an inhibitory protein that dissociates from NF-kappaB, was mediated by the mitogen-activated protein-kinase, p38. The results of this vitro work identify p38 as an intracellular target for high dose vitamin C. This is supported by the findings of Horton *et al.* (2001) who found that administration of 38 mg/kg vitamin C in conjunction with 27 U/kg of vitamin E and 41U/kg vitamin A, inhibited NF*k*B translocation from the cytosol to the nucleus and reduced inflammatory cytokine (TNFα, IL-1β, IL-6) secretion by cardiomyocytes following burn-trauma.

A perspective on the possible association between vitamin C and the pituitary gland, the adrenal glands and the immune system has been described by Kolb (1990). Cu-containing peptidydyl-glycine-alpha-amidiizating–monooxygenase, which is necessary for the formation of alpha-melanocyte stimulating hormone (αMSH) and dependent on ascorbate for its activity, is stored in the pituitary gland. In the event of an ascorbate deficiency, α-MSH formation is inhibited and a stressful situation will result in the increased binding of ACTH to the cells of the *zona fascicularis* and inner *zona reticularis* of the adrenal cortex. This stimulates the release of about 40-60% of the quantity of ascorbate from the adrenal cortex, activating adenylate cyclase and C-21-hydroxylase, and increasing the synthesis and secretion of glucocorticosteroids (Kolb, 1990).

The low molecular weight chemotactic cytokine, interleukin-8 (IL-8), produced by macrophages and stimulated by TNF and IL-1, is another important secondary mediator of the inflammatory response associated with tissue damage and glucocorticosteroid release. It has the ability to both attract neutrophils to damaged tissue and to activate them in response to elevated TNFα and IL-1 (Baggiolini, 1993) which are, in turn, known to stimulate steroid-hormone secretion by adrenocortical cells (Nussdorfer & Mazzocchi, 1998). *In vitro* neutrophil-mediated acute inflammation studies have reported elevated humoral IL-8 concentrations and shown that the administration of a neutralising antibody to IL-8, reduces neutrophil infiltration and neutrophil-mediated tissue injury (Mukaida, 1998). It is possible that this cytokine, which has been shown to be released into the bloodstream after prolonged and intensive, but not moderate exercise (Suzuki *et al.*, 2000), therefore has an important function in activating the hypothalamic- pituitary –adrenal axis and the accumulation of neutrophils in damaged skeletal muscle cells during prolonged eccentric, weight-bearing exercise such as downhill running. Inhibition of its release by vitamin C, would therefore reduce an exercise-induced inflammatory response as well as attenuate glucocorticoid release. This hypothesis requires investigation.

In a randomised, double blind, placebo-controlled study (Nieman *et al.*, 1997) supplementation with 1000 mg of vitamin C for 8 days to 2 groups of 6 distance runners, did not have significant effect on stress hormone concentrations, leukocyte subsets, IL-6, NK cell activity or lymphocyte proliferation following 2.5 hours of intensive running at 75-80% $\dot{V}O_2$ max (N=6). It is, however, a possibility that the duration and intensity of the exercise protocol was not adequate to elicit the oxidative stress and muscle cell damage which results from more prolonged eccentric exercise. Furthermore, dietary CHO intake during exercise may have been an extraneous variable modulating cortisol release into the blood stream via increments in blood glucose concentration in this work. High blood glucose would reduce ATCH activity and cortisol secretion (Tabata *et al*, 1991; Nehlsen-Cannarella, 1997; Nieman *et al.*, 1997), resulting in less release of AA from the adrenal gland into the circulation (Goralczyk, 1992). This may also have applied to the most recent work reported by Pederson *et al.* (2001) in which male recreational runners received either anti-

oxidants (500 mg vitamin C and 400mg vitamin E daily) or placebo 14 days before and 7 days after a 5% downhill 90-min treadmill run at 75 % VO_2 max. and exercise-induced changes in IL-1Ra, IL-6, creatine kinase and lymphocyte numbers did not differ significantly between the supplemented and non-supplemented groups. Further work into the efficacy of vitamin C supplementation in reducing markers of an inflammatory response to prolonged eccentric exercise in which the CHO status of the athletes is carefully standardised, is required.

2.3.7.2 Respondents to glucocorticoid release

Interestingly, cortisol appears to mediate at least some of its immunosuppressive effects through induction of the potent anti-inflammatory cytokine, IL-10 (Skinkai *et al.*, 1996). This soluble protein produced by helper T cells, macrophages/monocytes and B cells, which was originally referred to as cytokine synthesis inhibiting factor (CSIF), has both immunosuppressive and immunostimulatory properties. While increased circulating concentrations of this cytokine inhibit the release of TNFα and IL-1β and induce the production of IL-1Ra (de Waal *et al.*, 1991), inhibition of its bioactivity results in enhanced bacterial clearance, increased expression of proinflammatory cytokines and prolonged survival of mice challenged with *Klebsiella pneumoniae* (Greenberger *et al.*, 1995). Exposure of human monocytes to major group rhinoviruses (the main cause of the common cold) has also recently been reported to result in increased IL-10 production by these cells and down-regulation of their accessory functions (stimulation of T cell and NK cell function). These events (increased IL-10 production) have been proposed to be involved in the pathogenesis of upper respiratory tract infections with these viral pathogens (Stökl *et al.*, 1999). To my knowledge, however, no study has previously been undertaken in which the relationship between intensive exercise, vitamin C status, cortisol and IL-10 production is addressed. It was thus an objective of the studies presented later in this thesis to investigate this relationship.

A further respondent to glucocorticoid release is IL-1Ra, a specific inhibitor of IL-1 activity that acts by blocking the binding of IL-1 to its cell surface receptors. The

antagonist is secreted by several different cell types, including monocytes, neutrophils, macrophages, and fibroblasts. IL-1Ra is thought to be part of a naturally occurring mechanism that limits the extent of the potentially deleterious effects of IL-1. Several cytokines upregulate IL-1Ra production, including IL-6. This cytokine inhibitor has been shown to be elevated following prolonged exercise (Ostrowski *et al.,* 1999), but no study has to date investigated the effect of vitamin C supplementation on its production.

A "grey area' which thus presently exists in the state of knowledge regarding the role of vitamin C in attenuating infection risk following physical exertion, revolves around the possible effect of elevated circulating vitamin C concentrations on adrenal stress hormone production and consequent modulation of the production of IL-10 and IL-1Ra. This question has been addressed in the current study (Chapters 4, 5 and 6).

2.3.8 Exercise, vitamin C status and circulating adrenaline concentrations.

Adrenaline is known to possess anti-inflammatory and immunosuppressive properties (Galbo, 1983) and may accordingly also impact on the magnitude of the post-exercise "open-window" period (Pedersen and Ullum, 1994). During exercise adrenaline is released from the chromaffin granules in the adrenal medulla and plasma concentrations increase almost linearly with the duration of exercise and exponentially with intensity. The expression of beta-2 adrenoreceptors on T, B, and NK cells, macrophages and neutrophils provides the molecular basis for these cells to be targets for catecholamine signaling (Moore and Willoughby, 1995; Van der Poll *et al.*, 1996; Weiss *et al.*, 1996). Beta-2 adrenoreceptors on lymphocytes are linked intracellularly to the adenylate cyclase system for generation of cAMP as a second messenger (Hadden *et al.,* 1970). It has been shown that in most mammals, chromaffin granules of the adrenal medulla have the second highest concentration of AA (the adrenal cortex has the highest) [Dhariwal *et al.,* 1989]. In terms of a possible relationship between blood vitamin C concentration and catecholamine release, an enzyme in the chromaffin granules, dopamine beta-monooxygenase, requires AA *in vitro* to convert dopamine to noradrenaline in the catecholamine biosynthesis

pathway (Levine *et al.*, 1986). No known relationship between blood vitamin C concentration and adrenaline release, has, however, been described.

In conclusion, this review of the relevant literature reveals that the evidence in support of an anti-oxidant function of vitamin C during prolonged, aerobic exercise is strong, but that the effect of vitamin C supplementation on the mobilisation of vitamin C stores, pro-inflammatory cytokines and their natural antagonists and adrenal stress hormone release, requires further investigation. The effect of vitamin C supplementation on neuro-endocrine interactions and local inflammatory response are also open areas for future research some of which have been investigated in the studies described in the following chapters of this thesis.
