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Beta-Carotene and Exercise Performance: Effects on Race Performance, Oxidative Stress, and Maximal Oxygen Consumption.

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BETA-CAROTENE AND EXERCISE PERFORMANCE:
EFFECTS ON RACE PERFORMANCE, OXIDATIVE
STRESS, AND MAXIMAL OXYGEN CONSUMPTION

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Kinesiology

by
Kim Edward LeBlanc
B.S., University of Southwestern Louisiana, 1975
M.D., Louisiana State University Medical Center - Shreveport, 1978
December 1998
If you always do, what you've always done.
You'll always get, what you've always got.

Author Unknown
This work is dedicated to my wife, Mary Frances.
She has been the reason for success
in all my endeavors.
She has guided me through both good times and bad, and
reassured me when my confidence was lacking.
Her patience was unbelievable as was her understanding.
Even when there was a question of my sanity in pursuit
of yet another degree, she never
impeded my efforts to pursue my dreams.

This work is further dedicated to our three daughters:
Marie, Anna Mary, and Natalie
These girls are my joy and my pride.
Words could never express my love for them.
Without them, my world would be empty.
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Deep appreciation to Arnold Nelson, Ph.D., my major professor and faculty advisor. His calm manner and efforts to force me to "lighten-up" will always be remembered. Dr. Nelson's knowledge base seemed endless and continually one step ahead of my thoughts.

Eternal thanks to the runners/athletes who participated in this study. Even though this research required multiple blood draws and repeated runs at specified times and paces, we are still friends!

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Remembrance to my father, Peter Holden LeBlanc. His untimely death at the age of 45 in 1971 was certainly a tragedy. However, he left his mark on my life which has remained indelible and ever-present. I have always marveled at his pursuit of education and receipt of his Masters degree at LSU in 1955 (long before this was a common event). Finally, I may join him in receiving a graduate degree at Louisiana State University in Baton Rouge. My father loved LSU and would be proud that I have completed this work. Thanks dad. I love you.

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ABSTRACT

There is evidence in the literature that suggests a possible worthwhile role of antioxidants during athletic endeavors and exercise performance. Several studies have evaluated the antioxidant ability of beta-carotene and demonstrated a beneficial influence. However, these studies have investigated beta-carotene's effects within an antioxidant mixture which included other antioxidant vitamins. Consequently, this study attempted to isolate the antioxidant role of beta-carotene during exercise. Three parameters of beta-carotene influence were measured using eleven well-trained runners: serum malondialdehyde (MDA) concentration (a marker of free radical generation), 5000 meter race performance, and maximum oxygen consumption (VO$_{\text{max}}$). Subjects were tested using a double-blinded cross-over design. During the supplemental phase, each subject ingested 25000 IU beta-carotene daily.

The results indicated that the beta-carotene had no statistically significant effect on MDA levels or VO$_{\text{max}}$. However, there was a statistically significant improvement in 5000 meter race performance. In addition, 64% of the subjects noted a subjective benefit from the use of beta-carotene supplementation.

Therefore, results of this study indicate that beta-carotene can provide a beneficial influence on race performance in well-trained runners. However, this investigation did not furnish an adequate explanation of the mechanism by which this occurs.
CHAPTER 1
INTRODUCTION

The generation of free radicals species may represent the negative side of exercise. Data is accumulating which reveals an association between exercise and increased free radical formation. This increased production of free radicals coincides with oxidative tissue damage. This tissue damage will result in impaired functioning of various cellular and subcellular processes with a progression toward cell death. Any detrimental effects would be magnified in a physically active person. However, it must be kept in mind that there has been no definitively established link between cellular malfunctioning and/or death and free radical formation.

An exercising individual must increase the utilization of available oxygen to maintain adequate oxygenation to the tissues. As a consequence of strenuous exercise raising oxygen consumption, there may be an associated increase in free radical production, leading to lipid peroxidation and possible damage [1, 2]. Beta-carotene has been shown to possess the ability to quench singlet oxygen as well as functioning as an antioxidant [3, 4, 5]. This fat-soluble provitamin A has been demonstrated to decrease lipid peroxidation, attesting to its antioxidant ability [6]. These traits would permit beta-carotene to protect cellular membranes and tissues against oxidative damage.

As an antioxidant, β-carotene was used in a few studies to investigate if it could ameliorate exercise-induced lipid peroxidation and skeletal muscle damage. Unfortunately, because β-carotene was
supplied in a mixture containing other antioxidants, vitamin C and E, it is difficult to determine if any positive result can be ascribed to the beta-carotene or the other components. In one study, supplementation of 10 mg beta-carotene, 800 mg vitamin E, and 1000 mg vitamin C daily for 2 months increased the antioxidant potential of the blood glutathione system, counteracting exercise-induced GSSG (oxidized glutathione) elevation and significantly diminishing the indicators of muscle damage (plasma lactate dehydrogenase and creatine kinase) [7].

A similar study with a higher dose of 1000 mg vitamin E, 1250 mg vitamin C, and 37.5 mg beta-carotene failed to demonstrate any positive effect on muscle damage or lipid peroxidation indicators [8]. The authors speculated that their exercise regimen of downhill running at 65% maximal heart rate did not present enough metabolic challenge. Another study with the same vitamin mixture consumed daily for 5 weeks decreased lipid peroxidation (measured by serum malondialdehyde and breath pentane) at rest and following exercise at 60 and 90% VO\textsubscript{2 max} on a level treadmill [9]. Trying to assess oxidative damage to nucleic acids, the urinary output of 8-hydroxyguanosine was studied before and during three consecutive days of submaximal exercise, with and without antioxidant supplementation consisting of daily doses of vitamin E 553 mg, vitamin C 1000 mg, and beta-carotene 10 mg [10]. No significant differences were noted due to exercise or supplementation.

The equivocal results of the above described intervention studies stem from differences in methodology. The researchers used different tests, different levels of supplementation, and different intensities and

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durations of exercise. Nevertheless, there is promise in the antioxidant capacity of beta-carotene, which should be assessed as a single supplement. It is possible that the antioxidant effects of beta-carotene could serve as an ergogenic aid to the exercising athlete.

1.1 Project Rationale

Supplementation of beta-carotene could assist the body's defenses against oxidative damage. It has been demonstrated that humans are endowed with an antioxidant defense system to combat damaging free radicals. These defenses have evolved to aid the body in protecting itself from the oxygen-rich environment in which humans inhabit. Toxic derivatives of oxygen such as superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), the hydroxyl radical (OH*), and singlet oxygen (1O$_2$), are produced with each respiration. Enzymes are available to detoxify free radicals and are represented by superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX). These enzymes attempt to maintain the delicate balance between free radical generation and antioxidant defense.

There is a substantial amount of data which exists to suggest that these enzyme systems may provide adequate protection only during basal metabolic states. Exercise will cause an elevation of respiration and metabolism which may surpass the body's ability to defend itself against free radical bombardment. The sedentary individual may be able to rely on the body's endogenous defense mechanisms while the athlete may fall victim to inadequate protection. This would suggest that the exercising individual may benefit from antioxidant supplementation.
The carotenoid antioxidant, beta-carotene, was chosen for study as a possible ergogenic aid for the following reasons:

(1) There is existing data that has shown that adequate intake of beta-carotene may be lacking in some athletes (while the levels of vitamin E and C are satisfactory), i.e., marathon runners, weight lifters, swimmers, and cyclists [11]; A preliminary study performed by this researcher verified the fact that long distance runners (all running > 40 miles/wk) may have inadequate levels of beta-carotene. All three subjects had below normal levels of beta-carotene prior to beginning the study as noted by the following: subject 1 had a level of 14 μg/dl, subject 2 had a level of 15 μg/dl, while subject 3 had a beta-carotene level of 23 μg/dl (normal 35-250 μg/dl);

(2) Beta-carotene is not absorbed by laboratory animals, such as rats or mice. Therefore, it is quite difficult to study this provitamin A without the use of human subjects (as opposed to vitamin C and E which may be studied using various laboratory animals) [12];

(3) Beta-carotene is the best quencher of the highly reactive singlet oxygen [3]. Although vitamin E and C are able to quench this reactive oxygen species, their levels appear to be adequate and have been studied extensively. Due to the fact that beta-carotene levels may be unsatisfactory in athletes, the body’s defenses against singlet oxygen may be lacking and unprotected from oxidative stress.

(4) In addition to its ability to quench superoxide anions and singlet oxygen, beta-carotene has been shown to possess the ability to function as an antioxidant [4]. This antioxidant capacity protects cell membranes from chain-reaction lipid peroxidation;
(5) Beta-carotene has the ability to function at low concentrations and in conditions of low partial pressures of oxygen (seen within the periphery of tissues at the cellular and subcellular level) [4, 5]. This has not been definitively shown with other antioxidants;

(6) Several studies, cited above, have evaluated beta-carotene in combination with vitamin C and E. These investigations support the use of beta-carotene to decrease lipid peroxidation and oxidative damage;

(7) There are no existing data or studies on the use of beta-carotene alone as it relates to enhancing exercise performance and/or reducing oxidative damage;

(8) This study would attempt to determine if a five kilometer (5K) run will induce oxidative stress in well-trained runners. A study, such as the described, represents a fairly clear-cut investigation attempting to demonstrate whether or not beta-carotene behaves as an antioxidant in vivo.

(9) A pilot study performed by me provided evidence that the ingestion of beta-carotene improved 5K (3.1 miles) performance, improved $\text{VO}_{2\text{max}}$ and decreased thiobarbituric acid reactive substances (TBARS) levels in trained runners running $> 40$ miles/wk. All three subjects were 100% compliant as verified by diary and history.

The following table represents the TBARS levels and $\text{VO}_{2\text{max}}$ of three subjects who were adhering to a study protocol similar (but not identical) to the one undertaken for my dissertation. Each of the blood samples were obtained before and after a 5K run. The first was
obtained as a baseline standard, the second followed beta-carotene depletion, and the third represented samples obtained following a one month period of beta-carotene supplementation (25,000 international units/day).

Table 1. Pilot Study results: TBARS, VO\textsubscript{2}max, and Race Performance

<table>
<thead>
<tr>
<th>Subject</th>
<th>TBARS Level (Normal = &lt; 2.5 nmol/ml)</th>
<th>MAXIMAL OXYGEN CONSUMPTION (L/min)</th>
<th>RACE PERFORMANCE FOR 5000 METERS (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BASELINE Pre Post DEPLETION Pre Post SUPPLEMENTED Pre Post</td>
<td>BASELINE 55.6 SUPPLEMENTED 64.7</td>
<td>BASELINE 19:39 DEPLETION 19:57 SUPPLEMENTED 19:33</td>
</tr>
<tr>
<td>01</td>
<td>3.0 2.8 Pre 4.3 Post 4.1 Pre 2.7 Post 3.1</td>
<td>62.9 66.3</td>
<td>18:17 18:44 18:36</td>
</tr>
<tr>
<td>02</td>
<td>3.0 2.8 Pre 3.1 Post 4.0 Pre 3.4 Post 3.6</td>
<td>57.2 61.2</td>
<td>21:53 22:14 21:27</td>
</tr>
<tr>
<td>03</td>
<td>2.5 2.4 Pre 5.1 Post 5.1 Pre 3.3 Post 3.5</td>
<td>19:39 19:57 19:33</td>
<td>18:17 18:44 18:36</td>
</tr>
</tbody>
</table>

This pilot study provided results that were encouraging and suggested that benefit may be derived from the supplementation of beta-carotene. This would allow a speculative view that there may be an ergogenic enhancement by its use.
1.2 Problem Statement

It was the purpose of this study to evaluate the effects of beta-carotene on exercise performance, oxidative stress, and maximum oxygen consumption (VO$_{2\text{max}}$). This study will also determine if endurance exercise does induce oxidative stress in subjects made up of well-trained runner-athletes.

1.3 Hypothesis

In accordance with the above statements, the following hypotheses were tested:

1. During the running of a 5K distance, even well-trained runners will see an increase in biomarkers of oxidative stress. This will be identified by increases in serum MDA concentrations.

2. Oxidative stress is produced during a 5K race/run. This stress may be reduced by beta-carotene. As a result of supplementation, exercise performance will be enhanced, as measured by improved 5K race times, compared to the normal daily dietary intake of β-carotene in well-trained runners.

3. Supplementation with beta-carotene will reduce cellular and subcellular oxidative damage. Consequently, beta-carotene supplementation will improve maximum oxygen consumption.

1.4 Limitations

There are many factors that are called into play when evaluating athletic performance. This study investigated only well-trained runners who were dedicated and consistent in their training. This study did not evaluate the effects of work-related activities, environment, or rest on oxidative stress or exercise performance. Additionally, this
investigation was limited to one specific amount of beta-carotene supplementation per day. Lastly, no attempt was made to determine actual serum beta-carotene levels in any of the subjects, before, during, or after supplementation.

1.5 Definition of Terms

The following list will identify terms and abbreviations used throughout this text pertinent to this research:

Antioxidant: substance that reduces or inhibits oxidation
Beta-carotene (β-carotene): provitamin A, no recommended daily allowance established: typical intake is 3.0 mg/day
Beta-carotene supplementation: 25000 units/day (15 mg/day)
Exercise performance: time to completion of 5K race
Free radical: molecule or molecular fragment, such as an atom, or group of atoms, with an unpaired electron in its outer orbit
5K race: running 5000 meters at all-out effort
5K run: running 5000 meters at 85-90% effort
MDA: malondialdehyde
MDA and TBARS: biomarkers of oxidative stress, at times, these may be used interchangeably
Oxidation: any reaction in which electrons are transferred from one atom to another
Oxidative stress: transfer of electrons which may be harmful
Subjects: runners consistently running > 40 miles/week
TBARS: thiobarbituric acid reactive substances
$\text{VO}_{\text{max}}$: maximum oxygen consumption
2.1 The Chemistry of Free Radical Generation and Oxidative Stress

Chemical reactions occur not only in the environment, but in living organisms as well. No matter where they occur, all chemical reactions can be classified as either oxidation-reduction reactions or nonoxidation-reduction reactions. It is the former that concerns the body when discussing the production of free radicals.

One of the principal reactions involved in free radical formation is reduction-oxidation (red-ox) reactions. Electrons and electron-exchange are the basis for these red-ox events. Oxidation does not simply mean the addition of oxygen atoms, it is more generally applied to any reaction in which electrons are transferred from one atom to another. In this situation, oxidation refers to the removal of electrons, and reduction (the opposite of oxidation) means the addition of electrons. The electron-accepting compound will be reduced and the donor oxidized. Another way of expressing this relationship involves two atoms that form a covalent bond. An atom ending up with a greater share of electrons acquires a partial negative charge and is said to be reduced, while the other atom acquires a partial positive charge and is said to be oxidized. Thus, Fe\(^{2+}\) is oxidized if it loses an electron to become Fe\(^{3+}\), and a chlorine ion is reduced if it gains an electron to become Cl\(^{-}\).
2.1.1 Definition of Free Radicals

The term radical has been present for over 30 years. Although this term may have had a variety of specific meanings, it was originally meant to indicate a part of a molecule capable of independent existence [13]. Over the ensuing years, as chemical knowledge increased, the definition has evolved as well. In its present usage, the term free radical is used to signify a molecule or molecular fragment, such as an atom, or group of atoms, with an unpaired electron in its outer orbit [14].

Normally, each orbital has the ability to hold a maximum of two electrons. This is a common occurrence in nature as virtually all chemical bonds are made up of two electrons. Under appropriate conditions, these electron bonds may break either asymmetrically or symmetrically. When these bonds break symmetrically, a single electron stays with each fragment by a process called homolysis [15]. Homolysis forms radicals as in the case where \( X:Y \rightarrow X^* + Y^* \). (The dot is used to represent a radical, or the presence of one or more unpaired electrons). The unpaired electron is usually extremely exchangeable (i.e., reactive), which accounts for the chemical and physical reactivity of the radical species. (Incidentally, this radical property is the basis for the scavenging ability of antioxidants [16]).

As a result of the unpaired electrons, free radicals are extremely unstable and energy-rich. They are very reactive chemically due mainly to the fact that they are not in a stable spin state [17]. Free radicals readily give up or accept an electron to stabilize their unpaired electron. Radicals have the ability to quickly participate in chemical reactions because of electron exchange. These chemical reactions
enable the radical species to decrease its energy level and its potential to react. Usually, as a consequence of this reaction, the reactive radical is rendered less reactive. The end result will be a harmless species of an extremely stable radical state.

The half-life (or life span) of a free radical is extremely short. The level of reactivity will determine the survival of the radical species. The half-lives usually range from $1 \times 10^{-2}$ to $1 \times 10^{-6}$ seconds [18]. This reactivity arises from the previously mentioned unstable configuration with the unpaired electron. Once the radical participates in a chemical reaction, it is no longer reactive. There do exist, however, some radicals that maintain an extremely low level of reactivity and are referred to as stable radicals [19].

Radicals may have either positive or negative charges, or remain uncharged. It is the unpaired electron in the outer orbit that determines the radicals' charge. An uncharged radical is a molecule in which one of the electrons is excited and translocated to its own separate orbit. A positive or negative radical, on the other hand, has either lost or obtained a single electron. It is this unpaired electron in the outer orbit that results in the reactivity of the free radical species.

The radical species may be made up of various major atoms which determine its designation. These radicals may be referred to as: 1) a nitrogen (N)-centered radical; 2) a carbon (C)-centered radical; 3) a sulfur (S)-radical, or 4) an oxygen (O)-centered radical. This listing could continue in this manner dependent on the major atom being discussed. For the purposes of this dissertation, however, the term “free radical” will refer only to the oxygen-centered radicals.
It is not unusual for confusion to arise whenever the terms oxygen-free radicals and free radicals are used. Although there may exist a close association (and frequent confusion) between the two terms, reactive oxygen species and free radicals are not identical. The designation reactive oxygen species (ROS) refers to such entities as singlet oxygen, hydrogen peroxide, peroxide, hydroperoxide, and epoxide metabolites of endogenous lipids. These ROS contain chemically reactive oxygen-containing functional groups, but are actually not radicals. They may interfere with some tissue molecules and disrupt cellular functions, but this interference may not necessarily occur through radical reactions.

2.1.2 Sources of Free Radicals

Free radicals may be produced either endogenously or exogenously. They may be formed as a result of chemical reactions occurring within the cell or its organelles or may be influenced by exposure to conditions in the outside ambient environment.

Endogenous formation of free radicals may occur in one of two sites. Radicals may be produced within the cell and accordingly, influence various intracellular processes. Others may be formed within the cell and released into the surrounding environment. The generation of intracellular free radicals may result as a consequence of autooxidation and inactivation of small molecules such as reduced flavins and thiols [20]. A second source of endogenous free radicals are certain enzymatic activities include oxidases, cyclooxygenases, lipoxygenases, dehydrogenases, and peroxidases. Oxidases and mitochondrial electron transport systems are prime, continuous sources of intracellular, reactive oxygen species [20]. The generation of
free radicals may occur within the cytosol membranes and/or outer membranes, as well as any of the subcellular organelles such as the lysosomes, peroxisomes, mitochondria, and the endoplasmic reticulum. Some of the molecular species are represented by superoxide radicals (O$_2^-$) or hydroxyl radicals (OH$^-$). Superoxide radicals are a free radical state of oxygen formed from conversion of O$_2$ during normal respiration. Other reactive molecules such as hydrogen peroxide (H$_2$O$_2$) and singlet oxygen (^1O$_2$) are not free radicals but are certainly reactive and capable of causing cellular and subcellular damage [21].

The generation of free radicals may also occur within exogenous sources some of which would include tobacco smoke, certain pollutants, organic solvents, anesthetics, hyperoxic environments, and pesticides [20]. Certain medications as well as some of the aforementioned compounds may be metabolized to free radical intermediates that cause oxidative damage to certain tissues. Exposure to radiation may also result in the formation of free radicals within the exposed tissues [20, 22, 23].

2.3 Free Radical Chain Reactions

Free radicals may react with other molecules by several different mechanisms. There are three characteristic reactions of free radicals in biological systems: radical addition, electron transfer, and/or atom abstraction (Figure 1) [24]. Some reactions between two radicals create a neutral species:

$$\text{CH}_3^* + \text{CH}_3^* \rightarrow \text{CH}_3\text{CH}_3$$
Most commonly, however, this is not the case. The most common reactions that occur between radicals and neutral molecules are atom abstraction and addition \[25\]:

**Abstraction**  \[\text{ROO}^\cdot + \text{RH}_2 \rightarrow \text{ROOH} + \text{RH}^\cdot\]

**Addition**  \[\text{HO}^\cdot + \text{aromatic} \rightarrow \text{HO-aromatic}^\cdot\]

1. **RADICAL ADDITION OR COMBINATION**

\[\text{R}^\cdot + \text{X} - \text{Y} \rightarrow \text{X} - \text{Y} \]

\[\text{R}^\cdot + \text{X} - \text{Y} \rightarrow \text{X} - \text{Y} \]

2. **ELECTRON TRANSFER**

\[\text{R}^\cdot + \text{O}_2 \rightarrow \text{R}^\cdot + \text{O}_2^\cdot\]

3. **ATOM ABSTRACTION**

\[\text{R}^\cdot + \text{X} - \text{Y} \rightarrow \text{X} - \text{Y} + \text{RH}\]

Figure 1. Three characteristic reactions of free radicals in biological systems.
As noted earlier, free radicals are not in a stable spin state and as a result are very reactive chemically. This chemical reactivity enables the free radical to readily donate or accept an electron in order to obtain stability for its unpaired electron. As a consequence of the free radical reacting to either donate or accept a free electron, another free radical is produced; i.e.,

\[ R^\cdot + X \rightarrow R + X^\cdot \]

The aforementioned abstraction and addition reactions participate in the production of the second radical. This newly created free radical is also unstable and may also react with another molecule to produce yet another free radical; i.e.,

\[ X^\cdot + Y \rightarrow X + Y^\cdot \]

This process in which radical reactions repeatedly recur creating new, reactive radicals is referred to as a free-radical chain reaction \[23, 25\]. The onset of this type of chain reaction will lead to many deleterious unstabilizing reactions.

These free radical chain reactions are often referred to as autooxidation reactions and may be divided into three distinct steps: initiation, propagation, and termination \[26\]. The initiation phase marks the onset of the free radical chain reaction. This primordial radical may be formed as a result of normal metabolic processes. The propagation phase allows the reaction to continue. It is in this phase that several susceptible molecules are oxidized as a consequence of initiation. Each successively formed radical will seek to achieve stabilization and therefore react to either accept or donate an electron. In doing so, however, another free radical is formed which is likewise unstable and may react with another molecule to produce another
free radical, and so on. These reactions will continue until the chain is disrupted. This is accomplished by the final phase of *termination*. In this step, two radicals react together resulting in the formation of stable nonradical products, hence, the destruction of the free radicals [25]. This termination reaction of one free radical reacting with another free radical may be represented as follows:

\[ R^* + R^* \rightarrow R-R \]

This termination will occur whenever a substance, such as an antioxidant, gives up an electron, or an enzymatic process reduces the number of free radicals. In this manner, further chemical instability of the cellular milieu will not continue and further compromise any metabolic or enzymatic processes.

There are other ways in which these chain reactions of autooxidation may be halted. Free radical intermediates may also be stabilized or quenched (satisfied) by reacting with a compound that is not a free radical, but which becomes a very stable (free) radical once the free radical has added to it; i.e.,

\[ R^*_{(reactive)} + ST \rightarrow S^*A_{(stable)} \]

In chemical terms, the substances that react with free radicals to yield relatively stable, i.e., unreactive products, are known as spin traps (ST). Spin traps represent only some of the compounds that will react with free radicals and stabilize them. The relatively stable free radicals that are formed as a result of reacting with the spin traps are often referred to as spin adducts (S•A) [27].
2.3 Lipid Peroxidation and Cellular Membrane Damage

Free radicals may attack various compounds of a cell, including polyunsaturated fatty acids (PUFA), deoxyribonucleic acid (DNA) and proteins. All of these are important, but of particular note is the attack on PUFA. Cell walls and organelle membranes (such as sarcoplasmic reticulum and mitochondria) are made up of PUFA which may come under attack by free radicals. Due to the presence of a large quantity of PUFA in the membranes of mitochondria and the sarcoplasmic reticulum, these organelles are particularly susceptible to damage inflicted as a result of peroxidation. As free radicals attack the wall structure(s), there is destruction of some of the fatty acids that make up this membrane. Free radicals may continue along the cellular membrane traveling from one fatty acid to the next. As one free radical finds a pairing electron, another radical is created, which goes to the next fatty acid to find an electron, and so it continues. In this manner a chain of events is initiated known as lipid peroxidation. Lipid peroxidation is the most studied biologically relevant free radical reaction [28]. It may be generally defined as the “oxidative deterioration of polyunsaturated lipids” [29, 30].

Lipids and proteins are the major components of biological membranes. Both of these constituents may be damaged by lipid peroxidation and impair the ability of the membrane(s) to function. It is the injury to the cellular and subcellular membranes that are of particular importance to the exercising individual. This damage inflicted by lipid peroxidation is a cellular consequence of free radical formation. This oxidative deterioration known as lipid peroxidation involves the reaction of the PUFA and oxygen resulting in the
formation of the lipid radicals and hydroperoxides, which are semistable. This in turn promotes free radical chain reactions [31].

Before continuing this discussion of lipid peroxidation, some attention should be directed toward membrane lipids for a clearer understanding of this oxidative deterioration. Cellular membranes are usually made up of two mirror-images of each other and result in the formation of a so-called bilayered membrane. These membranes are mostly made up of lipids which are generally referred to as amphipathic molecules. Amphipathic molecules have two separate and distinct ends. One end has little affinity for water and is commonly referred to as the hydrophobic end. This end is in the formation of a "tail" and contains hydrocarbon regions which are directed toward the center of the bilayer. Opposite to the tail end is the so-called "polar" end. It is this end that is hydrophilic and is in contact with the cytosol internally and the surrounding cellular environment externally (Figure 2).

There is overwhelming evidence that the lipid bilayer is the basic structure of all cell and organelle membranes, with proteins being inserted in different parts of the bilayer [23]. In some ways, each half of the bilayer functions independently of the other. For example, in each half of the lipid bilayer, protein and lipid molecules can diffuse extremely quickly. However, exchange of lipid molecules between the two halves of the bilayer is rare [23]. This membrane fluidity is due to the presence of the unsaturated and polyunsaturated fatty acids side-chains in many of the membrane lipids. Damage to PUFAs tends to reduce the fluidity of the membrane. This is not without serious consequence as it is this fluidity which is known to be essential for
the proper functioning of biological membranes [23]. This decreased fluidity may result in cellular swelling, tissue inflammation, an inability to maintain ionic gradients, and perhaps, cell death.

Figure 2. Lipid bilayer structure of biologic membranes. Structures are schematic representations of phosphoglycerides (glycerophosphatides), which consist of fatty acid "tails" attached to polar "heads".
Initiation of a peroxidation chain reaction in a PUFA or membrane is due to the attack of any species with sufficient reactivity, i.e., free radical, to abstract a hydrogen atom from a methyl group (-CH-). The hydrogen atom has only one proton and one electron and removal of a hydrogen atom will leave behind an unpaired electron on the atom to which the hydrogen was originally attached [28]. In the instance of a methyl group, this would leave behind an unpaired electron on the carbon. -C=H-. The presence of a double bond in the fatty acid weakens the C-H bonds on the carbon atom adjacent to the double bond and makes the H• removal easier [23]. The carbon radical tends to be stabilized by a rearrangement of the molecular structure resulting in the production of a conjugated diene. It is the formation of conjugated diene bonds generated by abstraction of hydrogen atoms that initiates the peroxidation [18]. These conjugated dienes may react readily with a molecule of oxygen to produce a peroxy radical, R-OO•. These peroxy radicals possess the ability to abstract a hydrogen molecule from another lipid molecule, setting the stage for the propagation of this peroxidation of lipid molecules within the adjacent fatty acids within the cellular membrane, i.e., the chain reaction. As this peroxidation continues, the damage progresses along the membrane(s) until is terminated, as earlier discussed (Figure 3).

Lipid peroxidation, or autooxidation, as just described, possesses a self-destructive property resulting from free radical generation. This process also results in the formation of other cytotoxic products, such as lipid hydroperoxides, ROOH. Lipid hydroperoxides are produced when a peroxy radical combines with a hydrogen atom that it abstracts. One of the toxic products produced
are aldehydes (such as malondialdehyde) which may be used as a marker of the decomposition of ROOH [32, 33].

![Diagram of lipid peroxidation](image)

Figure 3. Scheme of the changes in the hydrophilic-hydrophobic balance of phospholipid molecules as a result of their oxidation.

Many different hydroperoxide species may be produced in the aftermath of lipid peroxidation. The number of different species is determined by the type of fatty acid that is involved in the autooxidation. The majority of fatty acids that undergo peroxidation in the cell membranes are linoleic acid, arachidonic acid, and docosahexaenoic acid. When these fatty acids undergo peroxidation, 2, 6, and 10 different hydroperoxide species are produced [34]. This allows for multiple products of degradation to be examined.

The damage induced in cellular and subcellular membranes via lipid peroxidation will impair the function of membranes as well as produce the aforementioned by-products such as hydroperoxides. The production of these by-products allow detection of the presence of lipid

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peroxidation by various methods and serves as biomarkers of this process. Detection of these biomarkers is important in documenting free radical formation and will be discussed in a later section.

2.4 Role of Oxygen in Free Radical Production

The human organism has evolved over many billions of years into a creature that requires the use of oxygen for its existence. The body has adapted to the presence of oxygen as necessary for respiration and energy production. Carbohydrates, fats, and proteins are nutritional substrates suitable for allowing hydrogen atoms to be removed during energy metabolism. These hydrogen atoms have electrons removed within the mitochondria of the cell, the actual site of energy production. These electrons are eventually passed on to molecular oxygen. Oxygen accepts hydrogen to form water and complete the process of cellular oxidation. During the transfer of electrons from hydrogen to oxygen, chemical energy is produced and stored in the form of high energy phosphate bonds. These high energy bonds are conserved in the molecules of adenosine triphosphate (ATP). This mechanism of the oxidation of hydrogen and the subsequent transfer of electrons to oxygen is referred to as the electron transport chain, or the respiratory chain, and as noted, occurs in the mitochondria. The vast majority of ATP synthesis occurs in this respiratory chain by oxidative reactions and is coupled with phosphorylation. This entire process is referred to as aerobic metabolism and provides energy for the functioning body. Although oxygen does combine with hydrogen to form water, its major role in energy metabolism is to serve as the final electron acceptor in the respiratory chain.
Molecular oxygen, however, may not be the "benign" substance that it would appear to be. Molecular oxygen, that is ground state oxygen, is a free radical in a technical sense as it is actually dioxygen. Dioxygen is, in actuality, a diradical with two unpaired electrons in the outer orbits. Fortunately, these two outer electrons exist in two separate orbitals. This is fortunate because these two electrons are thought to be spinning in the same direction. It is felt that electrons in the same orbit must have opposing spins [15]. In order for the dioxygen to add two electrons at the same time, the spin of one of the electrons would have to be reversed, which is an unlikely circumstance. Therefore, dioxygen is said to prefer to receive one electron at a time which converts it into a singlet oxygen molecule. When the unpaired electrons are balanced and spinning in opposite directions, these singlet states are not radicals or harmful. However, if energy is provided and the singlet oxygen enters into an excited state, reactions with biological molecules may result in oxidation of normal tissues. (Almost all biological molecules are in a singlet state which does not react with molecular ground state oxygen and therefore are not oxidized. Should singlet oxygen become energized, it will readily react with biological molecules and cause oxidative damage).

This seemingly would not be a problem if all of the oxygen was utilized by the body. However, it has been estimated that, regardless of activity level, up to 4-5% of the oxygen will form superoxide and ultimately hydrogen peroxide [35]. It would stand to reason that as the rate of oxygen consumption increases, so will the rate of superoxide formation and its resultant increase in free radical formation.
2.4.1 Production of Reactive Oxygen Species

As noted above, at the end of the mitochondrial respiratory chain, oxygen is reduced to form water. The respiratory chain is thought of as having four complexes, I, II, III, and IV (figure 4). The oxygen molecule, $O_2$, remains bound to complex IV of the mitochondrial respiratory chain (also known as ferrocytochrome c:oxygen oxidoreductase) until it is fully reduced to $H_2O$ by the transfer of four electrons and two protons. Further upstream in the complexes I, II, and/or III, there is leakage of single electrons, mainly from nonheme iron-sulfur proteins, leading to the partial reduction of $O_2$ to the superoxide anion ($O_2^-$) [36]. Superoxide is a radical, i.e., a chemical species with an unpaired electron. Since radicals tend to be reactive species because electrons like to pair up to form stable two-electron bonds, $O_2^-$ is referred to as one of the reactive oxygen species (ROS).

Figure 4. The sequential arrangement of the components of the electron transport chain, showing its division into four complexes, I, II, III, and IV.
It may be recalled that ground state oxygen is considered to be triplet oxygen (\( ^3\text{O}_2 \), although it is usually written as \( \text{O}_2 \)). This ground state oxygen may therefore be involved in the transfer of three electrons resulting in the formation of three different ROS. Superoxide anion has already been mentioned. Should this superoxide be further reduced by the addition of a second electron, a ROS will be produced which is not a radical. This second electron addition results in the formation of hydrogen peroxide, \( \text{H}_2\text{O}_2 \). The addition of a third electron leads to the formation of the hydroxyl radical (\( \text{HO}^* \)). \( \text{HO}^* \) is felt to be the most reactive and aggressive of the ROS [37].

In addition to the three aforementioned ROS, there is yet another type of ROS that may be formed, called singlet oxygen (\( ^1\text{O}_2 \)). Singlet oxygen may be formed from ground state triplet oxygen in the presence of a photosensitizer and light.

The leakage of electrons from the respiratory chain of the mitochondria to yield \( \text{O}_2^* \) occurs continuously during normal aerobic metabolism. It has been estimated that between 1-2% and 4-5% of all electrons that travel down the respiratory chain never make it to complex IV, but instead leak from the respiratory chain to form \( \text{O}_2^* \) and its dismutation product, \( \text{H}_2\text{O}_2 \) [35, 38]. There is some controversy as to whether the rate of electron leakage is not substantially lower under \textit{in vivo} conditions [36]. In addition to the mitochondrial and other cellular electron transport systems, there are further endogenous sources of superoxide production, some of which have been noted earlier in this paper. Some of these would be soluble oxidase enzymes, such as reduced nicotinamide-adenine dinucleotide phosphate.
(NADPH) oxidase in phagocytic cells (neutrophils and monocytes), and xanthine oxidases; epinephrine, and quinoid substrates, such as coenzyme Q₁₀ and vitamin K that undergo redox cycling; and the cytochrome P450 system [36, 39].

Under normal conditions of metabolism, the superoxide anion can be produced from all these various endogenous sources. Indeed, it has been estimated that each cell in the human body is exposed to about 10¹⁰ molecules of O₂⁻ per day, which would amount to approximately 1.75 kg (4 pounds) per year [39]. Thus, this superoxide is formed in substantial amounts and is not just a minor by-product of metabolism that is easily ignored or taken lightly. Once formed from the various endogenous sources, O₂⁻ may react further and dismutate resulting in the formation of H₂O₂ and O₂. These two entities, in the presence of catalytic amounts of iron or copper, can form HO⁻ in the "metal-catalyzed Haber-Weiss reaction". This reaction is the sum of two separate reactions: the first being the reduction of ferric or cupric ions (Fe³⁺ or Cu²⁺) by O₂⁻, and the second reaction of the reduced metal ions ferrous or cuprous, (Fe²⁺ or Cu⁺) with H₂O₂ to form HO⁻ (the "Fenton reaction"). Finally, HO⁻ and O₂⁻ may react to form ¹O₂, although this reaction probably does not occur very often in biological systems, mainly because HO⁻ is so reactive and will react at the site of formation, where O₂⁻ is not likely to be present. The sequence of reactions shown in figure 4 demonstrates that O₂⁻ can generate other ROS, including HO⁻, provided a redox-active metal catalyst is available.
2.4.2 Oxidative Damage

Deleterious free radical reactions and the generation of ROS may be collectively referred to as oxidative stress. This oxidative stress may cause oxidative damage to various macromolecules present in biological systems. As mentioned previously, HO• can damage cellular membranes and lipoproteins by lipid peroxidation. Additionally, proteins may be damaged by ROS, leading to structural changes or loss of enzyme activity. Oxidative damage to DNA may occur as well and has been reported to occur at a high rate under normal metabolic conditions. It has been estimated that the DNA in each cell in the human body is exposed to $10^4$ oxidative "hits" per day, leading to the formation of more than 20 different oxidative DNA lesions [39]. Many of these lesions are known to be mutagenic. Fortunately, there are a number of DNA repair enzymes that can remove these lesions. However, the repair systems are not perfect, and although they may remove 99% of the lesions, 1% still remains. This allows oxidative damage to continue within the DNA [39]. This has important implications concerning the body's defenses against ROS and oxidative stress, which will be addressed in a subsequent section.

As discussed, the ROS can cause oxidative damage to DNA, lipids, proteins, and carbohydrates. This oxidative damage to these biologic macromolecules may accumulate, not only with age, but may be increased in times of increased oxygen consumption, such as exercise. Even a single bout of exercise could promote oxidative damage and free radical-induced lipid peroxidation. With higher intensity exercise, more radical formation and subsequent damage will
occur, because higher intensity requires higher energy production which likewise increases oxygen consumption. Activity of the electron transport system and hydrogen peroxide formation [40].

Oxygen, therefore, is a double-edged sword. On the one hand, it is essential for our very existence. On the other hand, oxygen may accept electrons resulting in the formation of various deleterious products which are harmful to the body. This may be the “dark side” of exercise and oxygen consumption. As organisms that must respire and use oxygen, humans must be aware of both the benefits and detriments of the air we breathe.

2.5 Defense Against Oxidative Stress

There is a continual production of reactive oxygen species as a result of the aerobic nature of the body’s metabolism. Even under basal conditions, there will exist the ongoing production of free radicals and ROS. Hence, there is corresponding and continuous need for inactivation of these harmful products of aerobic metabolism.

A state of “oxidative stress” will exist whenever there is an imbalance of prooxidants and antioxidants in favor of the prooxidants [31]. Oxidative stress might also be defined as a state in which prooxidant reactions overwhelm the antioxidant defense of a tissue or organism. This is an unfavorable event and, similar to other such situations in the body, systems have been developed throughout evolution to combat this phenomenon. These systems have become the body’s defenses against oxidative stress and damage.

These protective networks are known as the antioxidant defenses. Halliwell and Gutteridge defined an antioxidant as “any substance
that, when present at low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate" [23]. These antioxidant defenses will therefore attempt to minimize or eliminate oxidative stress by creating an imbalance (or at least a balance) of antioxidants and prooxidants in favor of the antioxidants.

There are many antioxidants defenses in biological systems, some of which are listed in Table 2. Generally speaking, there are two types: enzymatic reactions, and non-enzymatic reactions. Some of the better known ones will be discussed.

Table 2. Antioxidant defenses in biological systems.

(Modified from [41])

<table>
<thead>
<tr>
<th>System</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enzymatic</strong></td>
<td></td>
</tr>
<tr>
<td>superoxide dismutase</td>
<td>CuZn, Mn, or Fe enzyme</td>
</tr>
<tr>
<td>catalase</td>
<td>peroxisomes, heme protein</td>
</tr>
<tr>
<td>Glutathione (GSH)</td>
<td>GPX</td>
</tr>
<tr>
<td>peroxidases</td>
<td>maintaining GSH levels</td>
</tr>
<tr>
<td>Glutathione (oxidized):</td>
<td></td>
</tr>
<tr>
<td>GSSG reductase</td>
<td></td>
</tr>
<tr>
<td>NADPH supply</td>
<td>NADPH for GSSG reductase</td>
</tr>
<tr>
<td><strong>Non-enzymatic</strong></td>
<td></td>
</tr>
<tr>
<td>alpha-tocopherol (vitamin E)</td>
<td>radical chain-breaking</td>
</tr>
<tr>
<td>β-carotene</td>
<td>singlet oxygen quenching</td>
</tr>
<tr>
<td>lycopene</td>
<td>singlet oxygen quenching</td>
</tr>
<tr>
<td>coenzyme-Q10</td>
<td>radical scavenger</td>
</tr>
<tr>
<td>ascorbate (vitamin C)</td>
<td>diverse antioxidant functions</td>
</tr>
<tr>
<td>glutathione (GSH)</td>
<td>diverse antioxidant functions</td>
</tr>
<tr>
<td>urate</td>
<td>radical scavenger</td>
</tr>
<tr>
<td>bilirubin</td>
<td>plasma antioxidant</td>
</tr>
<tr>
<td>plasma proteins</td>
<td>metal binding, e.g., ceruloplasmin</td>
</tr>
</tbody>
</table>
2.6 Enzymatic Mechanisms

The body is endowed with endogenous antioxidant enzymes, the most important of which are superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX). Each of these will be discussed in detail. Briefly stated however, superoxide dismutase catalyses the dismutation of superoxide to oxygen and hydrogen peroxide. (Dismutation is defined as a reaction(s) involving two identical molecules in which one gains what the other is losing; e.g., one may be oxidized and the other reduced). Catalase, in turn, converts hydrogen peroxide to water and oxygen. Glutathione peroxidase can remove hydroperoxides and hydrogen peroxide by consuming reduced glutathione to form oxidized glutathione. These enzymes are distributed within the cellular compartments in such a way as to complement each other by destroying radical species produced in the same cell compartments. These free radical-scavenging enzymes are the first line of cellular defense against oxidative injury [42].

These enzymes catalyze the following reactions:

**Superoxide dismutase**

\[ O_2^- + O_2^- \rightarrow O_2 + H_2O_2 \]

**Catalase**

\[ 2 H_2O_2 \rightarrow 2 H_2O + O_2 \]

**Glutathione peroxidase**

\[ H_2O_2 \rightarrow 2 H_2O \]

\[ ROOH \rightarrow ROH + H_2O \]
2.6.1 Superoxide Dismutase (SOD)

Superoxide dismutase (SOD) is an enzyme that was first discovered by McCord and Fridovich and serves to dismutate superoxide to form hydrogen peroxide and oxygen as shown in the above reaction on the preceding page [43]. Several forms of SOD exist in the cell. The manganese-containing SOD (Mn-SOD) is present in the matrix space of the mitochondria. The location of Mn-SOD is such that it is located close to the site where the superoxide radical is formed, i.e., the succinate dehydrogenase-cytochrome b segment.

Another SOD is the copper/zinc-SOD (Cu/Zn-SOD) which is present in the cytosol and mitochondria [44, 45]. Cu/Zn-SOD may also be found in the extracellular fluids in small amounts and, like that found in the cytosol and mitochondria, contains two copper and two zinc atoms [45]. All superoxide dismutases have at least one first transition series metal at the active site, i.e., Mn, Zn, or Cu (in animals), while bacteria have an additional type of SOD that requires Fe [46].

In mammals, SOD activity varies from one tissue type to another. The highest activity of SOD is found in the liver, followed by kidney, brain, adrenal glands, and surprisingly, heart [23]. Skeletal muscle has one of the lowest antioxidant enzyme levels in the body (levels similar to those found in the heart) [47, 48].

As noted from the above, SOD is able to convert superoxide into hydrogen peroxide which may be further decomposed by catalase and glutathione peroxidase. The catalytic activity of SOD has been shown to increase with increasing concentrations of superoxide and conversely to be inhibited by increasing concentrations of hydrogen peroxide.
peroxide [49]. In this manner, the enzymatic activity may be self-regulated to a certain degree.

2.6.2 Catalase (CAT)

The major function of catalase is to remove the hydrogen peroxide formed in the dismutation reaction of SOD. The hydrogen peroxide is decomposed to water. Catalase is a heme Fe$^{2+}$-containing enzyme which may be found in most cells and tissues. CAT is most abundantly found in the peroxisomes and probably serves to remove peroxide generated by the peroxisomal oxidase enzymes [50].

Peroxisomes contain oxidative enzymes, such as CAT, in high concentrations. Similar to the mitochondrion, the peroxisome is a major site of oxygen utilization. They contain one or more enzymes that use molecular oxygen to remove hydrogen atoms from specific organic substrates, designated here as R, in an oxidative reaction that produces hydrogen peroxide:

$$\text{RH}_2 + O_2 \rightarrow \text{R} + \text{H}_2\text{O}_2$$

Catalase utilizes the H$_2$O$_2$ generated by other enzymes in the organelle to oxidize a variety of other substrates by the "peroxidative" reaction which is designated as follows:

$$\text{H}_2\text{O}_2 + \text{R'H}_2 \rightarrow \text{R'} + 2\text{H}_2\text{O}.$$

(It should be borne in mind that CAT is found in the peroxisome and in the mitochondrion and other organelles as well [51, 52]).

There are tissue variations as to their enzymatic activity of CAT. The greatest activity is found in the liver while the skeletal muscle has the lowest. There are also interfiber differences of muscle CAT activity, much more so than with SOD [49].
Catalase does share its function of decomposing hydrogen peroxide to water with glutathione peroxidase (GPX). Both of these enzymes detoxify ROS by catalyzing the formation of hydrogen peroxide derived from superoxide. These two enzymes have different substrate affinities. At high concentrations of \( \text{H}_2\text{O}_2 \), organic peroxides are preferentially catalyzed by CAT. However, in the presence of low levels of hydrogen peroxide, organic peroxides are metabolized by glutathione peroxide \[18\].

### 2.6.3 Glutathione Peroxidase (GPX)

The antioxidant function of glutathione (GSH) is served via glutathione peroxidase (GPX) which catalyzes the reduction of hydrogen peroxide and organic hydroperoxides. GPX is located intracellularly and found in the cytosol and the mitochondrial matrix \[6\]. The removal of the hydrogen and organic peroxides is catalyzed by the selenium-dependent enzyme GPX, forming water and alcohol, respectively. This may be depicted in the following reactions:

\[
2 \text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2 \text{H}_2\text{O}
\]

or

\[
2 \text{GSH} + \text{ROOH} \rightarrow \text{GSSG} + \text{H}_2\text{O} + \text{ROH}
\]

In addition to requiring four selenium atoms, glutathione peroxidase uses two glutathione molecules (a tripeptide composed of glycine, cysteine, and glutamic acid) present in the reduced state (GSH). Glutathione, as well as cysteine, are thiols (R-SH). Thiols may be defined as sulfhydryls, i.e., any organic compound containing the -SH group. Thiols may act in aqueous and lipid environments as an antioxidant. Glutathione serves as a substrate for various enzymes.
including GPX, and during the above reaction. GSH becomes oxidized (GSSG). Each of the two glutathione molecules gives up a hydrogen from its sulfhydryl group (SH). A radical center is formed on the sulfur atom until two such radicals join to form a disulfide bond (GSSG).

GPX is a very important remover of hydroperoxide in the cell and, as such, plays a key role in inhibiting lipid peroxidation and, thereby, preventing damage to such things as RNA, DNA, and PUFA. The activity of GPX is high in the liver and erythrocytes; moderate in the brain, kidney, and heart; and low in the skeletal muscle [49]. However, some oxidative Type I skeletal muscle fibers, such as that found in the soleus, may exhibit GPX activity which approximates the levels found in cardiac muscle [53].

Once glutathione becomes oxidized to form glutathione disulfide (GSSG), it no longer possesses any of its protective antioxidant properties. The cell must therefore regenerate reduced glutathione (GSH) for it to be available for adequate functioning of GPX. It is in this regard that glutathione reductase is known for its function. Although not classified as an antioxidant on its own accord, glutathione reductase (GR) is essential for normal antioxidant function in the cell [49]. The cell regenerates reduced glutathione in a reaction catalyzed by glutathione reductase using nicotinamide adenine dinucleotide phosphate (NADPH) as a source of reducing electrons. This reaction takes place simultaneously with GPX, thus providing a redox cycle for the regeneration of GSH (figure 5). Thus, NADPH indirectly provides electrons for the reduction of hydrogen peroxide. Additional enzymes, such as SOD and CAT, catalyze the
conversion of other toxic oxygen intermediates to harmless products (figure 6).

![Figure 5](image)

**Figure 5**
Glutathione-mediated reduction of hydrogen peroxide by NADPH.

![Figure 6](image)

**Figure 6**
Actions of antioxidant enzymes.

### 2.7 Non-enzymatic Mechanisms

There are other mechanisms which are available to counteract the assault on the body by free radicals. Basically these mechanisms are compounds which are found in the food in the dietary intake. There are many such compounds which are known to exist. These substances do not function in an enzymatic fashion as that previously described, but instead function without the use of enzymes. The most well known and most investigated of these are the fat-soluble vitamins E and beta-carotene, and the water-soluble vitamin C. Another compound that has also been undergoing rather extensive investigation and should be considered within this context is
ubiquinone, which is also known as coenzyme \( \text{Q}_{10} \) (CoQ\(_{10} \)). CoQ\(_{10} \) is actually not a vitamin, but has been referred to as a "vitamin-like substance". These four compounds will be the subject of the following discussion on the non-enzymatic mechanisms of defense against oxidative stress.

### 2.7.1 Vitamin E (tocopherols)

Vitamin E refers to a family of related compounds called tocopherols. The molecule is highly lipophilic and resides almost exclusively in the various membranes in the cell. The most abundant and active isomer is alpha-tocopherol. As a result of its lipid solubility, it is an integral part of the structure of cell membranes.

Vitamin E is a fat-soluble antioxidant capable of protecting polyunsaturated fatty acids from lipid peroxidation by interrupting free radical reactions that otherwise can cause membrane damage in subcellular organelles and within the cell wall itself.

The major role of vitamin E is to serve as a chain-breaking antioxidant to neutralize free radicals and scavenge singlet oxygen as well as functioning as a membrane stabilizer [54, 55]. It is a chain reaction-breaking antioxidant because it quenches the intermediate in the free radical chain reaction (figure 7). By reacting with a free radical, the tocopherol molecule is converted into the tocopheroxyl radical, which can be reduced back to tocopherol by either vitamin C or glutathione.

Vitamin E is also able to directly scavenge most species of free radicals including superoxide, hydroxyl radical and lipid peroxides [56]. It will release one of its own electrons satisfying the missing electron within the free radical which will lead to an inhibition of lipid
Figure 7. Reaction of vitamin E with lipid radicals.

Peroxidation and formation of a so-called "inhibitor" radical. This inhibitor radical has low reactivity and will ultimately react with itself or with another peroxyl radical to form nonreactive degeneration byproducts [56]. In this manner, the free radical chain reaction is terminated.

Another important function of vitamin E is as a structural membrane stabilizer. It has the ability to increase membrane microviscosity and decrease passive permeability to low molecular weight substances [57]. This effect of vitamin E would be to increase the orderliness of membrane lipid packing, in effect straightening bent
unsaturated phospholipids and thereby allowing for a closer and more functional lipid bilayer. It has been suggested by some investigators that the major reason that vitamin E is able to protect against exercise-induced muscle damage lies in its membrane stabilizing ability rather than its antioxidant function [55, 58].

The physiologic function of vitamin E, therefore, is its role as an antioxidant and scavenger of free radicals, thus preventing damage to PUFA in cell membranes and its function as a membrane stabilizer. It is one of the primary factors in our defense against oxidative stress and subsequent damage.

2.7.2 Beta-Carotene

There are over 600 carotenoids that have been identified in nature. Carotenoids represent a very large group of substances with many varying structural characteristics and biological activities. They are found as pigments in green leafy vegetables and many colored fruits. The major and most nutritionally active carotenoid is beta-carotene which comprises 15-30% of total serum carotenoids [59]. Beta-carotene is considered a provitamin A, and is one of about 50 of the carotenoids that exhibit provitamin A activity. Beta-carotene plays a role in free-radical biology, having the ability to act as a quencher of the highly reactive singlet oxygen (1O2) formed during normal oxygen consumption and block some free-radical mediated reactions [3, 59]. There have also been reports that it is able to act as an effective antioxidant as well [4].

As noted, beta-carotene is a provitamin of vitamin A. Although vitamin A itself has not been shown to have any antioxidant effects,
this is not true of beta-carotene. This particular carotenoid has been shown to deactivate singlet oxygen mainly by physical quenching as displayed in the following reaction:

\[ ^1\text{O}_2 + \beta\text{-carotene } \rightarrow ^3\text{O}_2 + \beta\text{-carotene}^* \]

This energy-transfer process produces ground state molecular oxygen and the carotenoid triplet that, in turn, decays rapidly to its ground state. Thus, harmful chemical reactions of singlet oxygen with surrounding compounds are prevented [4]. The ability of certain carotenoids to quench \(^1\text{O}_2\) is related to the number of conjugated double bonds, with a maximum protection exhibited with nine or more double bonds [60]. This is one of the very important roles of beta-carotene which possesses eleven double bonds.

There has been other evidence, however, that has suggested that there may be another antioxidant role played by beta-carotene. The possibility of beta-carotene having an antioxidant property other than that of quenching singlet oxygen was suggested as long ago as 1932 by Monaghan and Schmitt [61]. Until recently, there has been no clear understanding of the mechanism by which beta-carotene accomplished this. Burton and Ingold conducted a series of experiments in an attempt to demonstrate that beta-carotene could function as an antioxidant in a biological system where there was an absence of singlet oxygen [4]. In their experiment, they placed beta-carotene into tetralin in chlorobenzene. These substances are known to lead to the formation of "substrate-derived radicals". In this system, no singlet oxygen was present. Interestingly, their results indicated that
beta-carotene was neither a peroxide-decomposing preventive antioxidant nor a conventional chain-breaking antioxidant [4]. This was evident under normal laboratory conditions, which consisted of temperatures of 30° centigrade and oxygen pressure of 760 torr. However, in living organisms, the partial pressure of oxygen is only about 20 torr. The tissues further in the periphery would have even lower partial pressures. This would be particularly true of organelles such as the mitochondria that are within the cells and are actively metabolizing oxygen. The results of Burton and Ingold bear this out. Their data revealed that “beta-carotene, particularly when it is present at rather low concentrations, is a very effective antioxidant.” This mechanism has no relationship to its effect as a quencher of singlet oxygen. From their study, it appeared that beta-carotene was able to reduce peroxyl radicals at low partial pressures of oxygen, i.e., a chain-breaking type of action. Their findings have been supported by other research in this area [5, 60, 62, 63].

Supporting evidence for this ability of beta-carotene to function as an effective antioxidant was also presented by Stocker et al [64]. They compared the antioxidant properties of beta-carotene and alphatocopherol at low oxygen pressures of 20% and 2% (150 torr and 15 torr respectively). Their results demonstrated that beta-carotene does have an antioxidant effect at low partial pressures. This was also substantiated by Vile and Winterbourn [65] as well as Kennedy and Liebler [5]. These investigators also provided evidence that showed the same antioxidant effect of beta-carotene at low partial pressures of oxygen.
With the above information, it therefore seems that beta-carotene is able to not only function as a quencher of singlet oxygen (which itself is not a free radical) but may also serve to function specifically as an antioxidant at the lower partial pressures of oxygen that are present in the periphery of the tissues. It would appear that the mechanism of beta-carotene’s antioxidant actions relates to the formation of an inhibitor radical. An inhibiting, carbon-centered radical is probably formed by the addition of an ROO• (and, perhaps also of an R•) radical to the conjugated system of β-carotene [4]. This would be particularly beneficial at the subcellular level of the organelles, such as the mitochondria. Since the oxygen pressures are usually in the order of a range that is below 20 torr, these observations suggest that β-carotene has an important role as an antioxidant in these environments.

Unfortunately, there has been only one study that has demonstrated the antioxidant effect of carotenoids in humans. This was a study that involved placing a study group on a carotenoid-free diet for two weeks and then repleting them with β-carotene. These investigators were able to demonstrate a decrease in lipid peroxidation [66]. Although this is the only study that has been done in this manner, there is general agreement that what was demonstrated and discussed here and in preceding paragraphs is, in fact, true. Therefore, it seems that beta-carotene does have an antioxidant effect at low levels of oxygen.

In summary, beta-carotene is a readily available provitamin. It is one of many carotenoids that occur in nature. It has a particular role
in quenching singlet oxygen, one of the reactive oxygen species formed during normal respiration. In addition to this function, beta-carotene has an antioxidant action. This is noted at the low partial pressures of oxygen that are found in the tissues at the cellular level. An antioxidant effect, coupled with its ability to quench singlet oxygen, enables beta-carotene to be an important and necessary nutrient in the aerobic and exercising body. It will diminish the damaging effects of free radicals and reactive oxygen species on the cellular and subcellular structures.

### 2.7.3 Vitamin C (ascorbate)

Vitamin C, also known as ascorbic acid or ascorbate, is a well-known water soluble vitamin. It is able to act as a cofactor in metabolic reactions and is involved in many biological activities, including the formation of collagen, neurotransmitters, and certain hormones. This particular vitamin requires regular ingestion as the storage capacity in the body is limited. Ascorbic acid is known to act as an antioxidant both in vitro and in vivo. Its antioxidant capabilities are carried out either directly or indirectly.

Vitamin C may directly quench, or satisfy, free radical species by reacting with superoxide anion and/or by stabilizing the hydroxyl radical (OH•). The vitamin scavenges aqueous superoxide and hydroxyl radicals and acts as a chain-breaking antioxidant in lipid peroxidations. In this regard, the vitamin C acts as a potent reducing agent possessing the ability to reduce oxygen-, nitrogen-, and sulphur-centered radicals. Ascorbate (AH) is able to react with OH• and produce an “inhibitor” radical. An example of this reaction is as follows:

42
AH + OH• -----> H₂O + A•

This allows the hydroxyl radical to be reduced and prevent free radical damage from taking place.

The effectiveness of ascorbate as an antioxidant is enhanced by the existence of a mechanism for the regeneration of ascorbate (AH) from its oxidation products, A• [71]. These are reactions in which glutathione (GSH) and glutathione disulfide (GSSG) participate as depicted in the following:

\[
\begin{align*}
2 \text{A•} + \text{H}^+ & \rightarrow \text{AH} + \text{A} \\
2 \text{A•} + 2 \text{GSH} & \rightarrow \text{GSSG} + 2 \text{AH}\cdot \\
\text{A} + 2 \text{GSH} & \rightarrow \text{GSSG} + \text{AH} + \text{H}^+ \\
2 \text{A•} + \text{NADH} + \text{H}^+ & \rightarrow 2 \text{AH}\cdot + \text{NAD}^+
\end{align*}
\]

Vitamin C may also act indirectly as an antioxidant via regeneration of reduced vitamin E, renewing its ability to again act as an antioxidant. This indirect action regenerates the active form of membrane-bound vitamin E which will allow protection of lipid membranes from peroxidation (Figure 8).

\[\text{Figure 8. Regeneration of tocopherol (E) from tocopherol radical (E•) by ascorbic acid (C).}\]

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The hydrophilic ascorbic acid, which is located in the aqueous phase of the cell, is unable to scavenge lipophilic radicals found within the lipid region of the membranes. However, ascorbic acid can act as a synergist with tocopherol [70, 72]. This synergism will be discussed later in detail within this paper. Sufficient to say at this juncture that there is some disagreement of whether this synergistic relationship actually exists. Some reports support it [70, 73, 74], while others do not [75].

Before leaving the topic of vitamin C as one of the non-enzymatic mechanisms of antioxidant defense, mention should be made of the ability of ascorbate to act as a prooxidant. The ability of ascorbate to serve as a biological reductant accounts not only for its antioxidant properties but also for its ability to catalyze free radical reactions [68, 70, 76, 77].

In the presence of redox-labile metals, a marked instability and prooxidant behavior of ascorbate is observed [76]. It is generally agreed that the prooxidant activity of ascorbate is derived from its ability to reduce transition metals, Fe^{3+} or Cu^{2+}, by a one-electron transfer mechanism, and also to reduce O_2, by a two-electron mechanism as shown by the reactions below [71]:

\[
\begin{align*}
\text{AH}^* + \text{Fe}^{3+} \text{ or } \text{Cu}^{2+} &\rightarrow \text{A}^* + \text{Fe}^{2+} \text{ or } \text{Cu}^+ \\
\text{AH}^* + \text{O}_2 + \text{H}^+ &\rightarrow \text{H}_2\text{O}_2 + \text{A}
\end{align*}
\]

These reduced products can then result in the production of superoxide and hydroxyl radicals as follows:

\[
\text{Fe}^{2+} \text{ or } \text{Cu}^+ + \text{O}_2 \rightarrow \text{O}_2^* + \text{Fe}^{3+} \text{ or } \text{Cu}^{2+}
\]
\[ \text{Fe}^{2+} \text{ or Cu}^+ + \text{H}_2\text{O}_2 \rightarrow \text{OH}^- + \text{HO}^+ + \text{Fe}^{3+} \text{ or Cu}^{2+} \]

With current available knowledge, it would appear that the antioxidant properties are much more prominent than the latter effects. It is unlikely that ascorbic acid acts as a prooxidant \textit{in vivo} under normal physiological conditions [70].

### 2.7.4 Coenzyme Q₁₀ (ubiquinone)

Coenzyme Q₁₀ (CoQ), also known as ubiquinone, is a well known component of the mitochondrial respiratory chain (figure 4) [78].

Energy-rich molecules, such as glucose or fatty acids, are metabolized by a series of oxidation reactions ultimately yielding carbon dioxide and water. The metabolic intermediates of these reactions donate electrons to specialized coenzymes, nicotinamide adenine dinucleotide (NAD⁺) and flavin adenine dinucleotide (FAD), to form the energy-rich reduced enzymes, NADH and FADH₂. These reduced coenzymes can, in turn, each donate a pair of electrons to a specialized set of electron carriers, collectively called the electron transport chain (figure 9). As electrons are passed down the electron transport chain, they lose much of their free energy. Part of this energy can be captured and stored by the production of adenosine triphosphate (ATP) from ADP and inorganic phosphate (Pᵢ). This process is called oxidative phosphorylation. The remainder of the free energy not trapped as ATP is released as heat [79].

Coenzyme Q is a quinone derivative with a long isoprenoid tail (the ubiquinone moiety with 10 isoprene units is designated as coenzyme Q₁₀). It is also called ubiquinone because it is ubiquitous in biological systems. CoQ is the simplest of the electron carriers and is
a small hydrophobic molecule dissolved in the lipid bilayer of the mitochondrial inner membrane and in other cellular endomembranes [79, 80]. A quinone (Q) can pick up or donate either one or two electrons, and it temporarily picks up a proton from the medium along with each electron that it carries (figure 10). Coenzyme Q can accept hydrogen atoms both from FMNH₂, produced by NADH dehydrogenase, and from FADH₂, which is produced by succinate dehydrogenase and acyl CoA dehydrogenase [79].

Recent research has demonstrated that approximately 30% of all CoQ takes part in its role as a coenzyme in the electron transport chain. The remaining 70% of its activity takes place as an antioxidant in most cells and tissues and their organelles [81, 82, 83, 84]. The role of CoQ, both in formation of free radicals and in the cell's protection against them, i.e., the antioxidant function, appears well established [81, 85, 86, 87]. The phenol-ring structure has the antioxidant
properties of the molecule, whereas the carbon chain (tail) is responsible for transport and retention of the molecule within membranes [81].

Figure 10. Quinones. Each of these electron carriers in the respiratory chain picks up one H+ from the aqueous environment for every electron it accepts, and it can carry either one or two electrons as part of a hydrogen atom (yellow). When it donates its electrons to the next carrier in the chain, these protons are released. In mitochondria, the quinone is ubiquinone (coenzyme Q), shown here.

Recent investigations have suggested that there exists an interrelationship between the lipophilic CoQ and lipophilic vitamin E. Reduced CoQ is continuously formed by mitochondria to regenerate vitamin E from its radical form, the alpha-tocopherol radical [88, 89]. In fact, it has been shown that CoQ is a more potent radical quencher, with estimates of CoQ having 10 times the antioxidant activity as vitamin E [88].

Under normal physiological situations, most tissues in the human body predominantly exist in the reduced state. It is known that reduced CoQ is continuously formed by the respiratory chain within
the mitochondria (figure 11). Reduced ubiquinone and reduced antioxidant compounds are prerequisites for proper antioxidant function.

\[
\begin{align*}
NADH_2 & \rightarrow \text{mitochondrial e}^- \\
& \rightarrow \text{quinol formation} \\
& \rightarrow \text{reduced vitamin E} (E-OH) \\
& \rightarrow \text{e.g., reduced ascorbate or other cycling antioxidants} \\
\end{align*}
\]

Figure 11. The significance of electron generation by mitochondria and the coenzyme Q\textsubscript{10} transfer system.

It is this reduced state of CoQ that will react with free radicals and fulfill their antioxidant role [81, 82]. These reactions may be described as follows:

1. Superoxide radical:
   \[
   \text{QH}_2 + 2 \text{O}_2^- \rightarrow \text{Q} + \text{H}_2\text{O}_2 + \text{O}_2
   \]
   From previous discussion, it was noted that hydrogen peroxide is metabolized in many different ways based on enzymatic processes.

2. An organic free radical from a lipid, protein, or nucleotide (as symbolized by R\textsuperscript{•}):
   \[
   \text{QH}_2 + 2 \text{R}^\cdot \rightarrow \text{Q} + 2
   \]

48
(3) Peroxyl radicals:

\[ \text{QH}_2 + 2 \text{ROO}^- \rightarrow \text{Q} + 2 \text{ROOH} \]

As can be seen from this discussion, CoQ has a very definite role in the cell's antioxidant defense. As discussed, CoQ's role, similar to other antioxidants, is based on the notion that the antioxidant is in a reduced state and has the ability to donate electrons to a radical to quench it and remove its harmful potential. Comparable with the other previously discussed nonenzymatic mechanisms of antioxidant defense, CoQ has a role to allow the exercising individual a combatant against the bombardment of free radicals on tissues and cellular structures.

2.8 Exercise and Free Radical Generation

Although exercise has been shown to have many benefits, there have been few well-controlled prospective studies that actually support the notion that long-term exercise promotes longevity or protection from disease. Some researchers have speculated that the generation of free radicals may represent the "dark side" of physical activity. The suggestion being that because of the damage inflicted by free radicals the purported benefits of exercise may be negated.

Oxidative stress appears to be related to the intensity of the exercise. Exercise increases the rate of oxygen consumption and flux of electrons through the mitochondrial electron transport system (site of ATP production). Intuitively, it would seem reasonable that free radical formation would be enhanced to a greater extent during exercise when compared to the resting state. During exercise, when
oxygen consumption is elevated 10 to 15 times above rest, free radicals may be formed at a correspondingly higher rate.

2.9 Exercise-induced free radical formation

Generally speaking, the mitochondria located in skeletal muscle are a major producer of free radicals, with oxygen-centered radicals in particular being formed during physical activity [10]. There have been numerous studies which indicate that free radical processes, such as lipid peroxidation, were elevated during exercise. Perhaps the most frequently quoted data in support of a role of free radicals in exercise-induced damage to skeletal muscle is that of Davies and co-workers [90]. This data demonstrated that exercise to exhaustion in rats resulted in decreased mitochondrial respiratory control, lost sarcoplasmic reticulum integrity, increased lipid peroxidation and increased free radical generation as shown by electron spin resonance studies.

As a consequence of increased oxygen consumption during even a single bout of exercise, it is reasonable to assume that lipid peroxidation may occur within cells as a result of formation of free-radicals. In fact, it has been estimated that for every 25 oxygen molecules reduced by cytochrome oxidase during respiration, there is the concomitant production of one free radical [91]. As noted earlier, electron transport through the mitochondria may result in free radical formation and reactive oxygen species. Consistent with this premise is the fact that the production rate of hydrogen peroxide within the mitochondria has been linked directly to energy production within the electron transport system [40]. There have been other researchers that
have provided evidence for the production of free radicals during
exercise [9, 10, 42, 47, 92, 93, 94, 95].

Within the framework of these studies, it is possible to propose
a theoretical model for exercise-induced free radical formation.
Prooxidant reactions may be initiated when (1) the electron flow
through the cytochrome chain is high, (2) hypoxic tissues provide an
abundance of hydrogen ions to react with the increased levels of
superoxide produced, and (3) hypoxia leads to a freeing of transition
metals, such as Fe, Cu, Mg, to catalyze radical formation [96].

In spite of the data cited in the preceding paragraph, not all
studies have produced similar results. There are various reports that
have not demonstrated an association between exercise and oxidative
stress. A study which monitored both plasma and skeletal muscle
markers of free-radical-mediated damage following maximum eccentric
and concentric exercise, examined the potential role of free radicals in
exercise-induced muscle damage. The results of this study provided no
support for the involvement of oxygen free radicals in exercise-induced
muscle damage [97]. This particular study by Saxton et al. was done in
such a manner as to induce skeletal muscle damage without
substantial elevation of the metabolic rate. It was felt unlikely that
the metabolic stress encountered when an isolated muscle performs a
bout of discrete eccentric muscle actions, with rest periods between
repetitions, is of a similar order of magnitude to that which occurs
with running where the potential exists for generation of oxygen free
radical species in other tissues besides active skeletal muscle.
Although there may not be a large quantity of oxygen consumed with
repetitive exercise, it was felt that concentric work incurs greater
oxygen cost than comparable eccentric work but produces minimal damage to the muscle. Another study searched for evidence of oxidative stress following a triathlon race. This study utilized competitors who were trained for the event. At the completion of the triathlon, these investigators were unable to demonstrate any evidence of oxidative stress [98]. One other study examined trained rowers and subjected them to high intensity rowing. Total daily training time was 65 minutes at 70% VO$_{2\text{max}}$ and 38 minutes at $\geq$ 90% maximal VO$_2$ for a period of four weeks. There was no apparent oxidative stress or muscle damage produced from this specific activity [99].

Nevertheless, there is substantial evidence to support the production of free radicals during exercise. Evidence to support the role of free radicals as a cause of skeletal muscle damage, however, is less clear and remains elusive. There are many confounding factors involved in finding a resolution to this question. First, there is a major difficulty in obtaining comparable data. The aforementioned studies vary in the mode of exercise performed, fitness of the subjects, intensity of the exercise and the length of the exercise bout. For example, weightlifting type exercises are mostly anaerobic type activities. Although weightlifting may be able to produce skeletal muscle damage, it may not be of sufficient aerobic intensity to utilize the mitochondrial electron transport chain and produce oxidative stress. It is also questionable to assume that damage can be caused by a single mechanism. Just because ROS do not cause damage during anaerobic work does not mean they are inactive during aerobic work. Another example would be that low-intensity aerobic activities in
trained individuals may not produce oxidative stress and/or overcome the adaptive training effect so that no evidence of oxidant stress would be noted. Another shortcoming is the methods employed to document exercise-induced free radicals. This will be addressed in the following section.

2.10 Markers of exercise-induced tissue damage

Various by-products of oxidative damage induced by exercise may be measured to verify that oxidative stress has occurred. The most accurate methods (but least available) are those that measure free radicals directly, such as electron spin resonance (ESR) spectroscopy or electron paramagnetic resonance (EPR) spectroscopy. This technique is able to measure the actual amount of free radicals formed. The most commonly used methods for determining oxidative stress, however, have involved indirect measurements of a variety of biomarkers. Depending on the specific marker, they may be measured in the blood, urine, or in expired gases [9, 23]. Such biomarkers are utilized because they are readily available and because they measure the by-products of oxidative damage. Some of the most commonly used markers are ethane and pentane, conjugated dienes, malondialdehyde (MDA), thiobarbituric acid reactive substances (TBARS), and serum enzymes.

The methods named above have been used to verify and quantitate the existence of exercise-induced skeletal muscle damage and lipid peroxidation. The results of any study utilizing one of these techniques are called into question due to the methodology chosen to assess the biomarkers of oxidative stress. As with all scientific
endeavors, the use of the most accurate and reliable method is always tempered by such important considerations as cost, availability, resources, time constraints, feasibility and ease of use. Certainly all researchers would relish the idea of using the best means possible at all times and in every situation. Regrettably and realistically, this is not always possible. As these are the most commonly used methods of determining the presence of oxidative and/or skeletal muscle damage, the positive and negative reasons to support their use will be addressed. Ultimately, the choice of the most appropriate method to utilize must be individualized.

2.10.1 Breath ethane and pentane

Oxidation of polyunsaturated fatty acids (PUFA) results in the production of ethane and pentane which may be detected via breath analysis. Both of these gases are formed as a result of degradation (peroxidation) of PUFAs. Pentane is derived from the omega-6 family of PUFAs (such as linoleic acid), while ethane is derived from the omega-3 PUFA family (such as linolenic acid) [100]. These expired compounds may be utilized as an index of lipid peroxidation and may be applied as a quantitative measure in vivo using gas chromatography [101, 102].

One of the valuable qualities of this methodology is the fact that this testing procedure is a noninvasive in vivo measure of peroxidation of lipids. This test is readily performed and easily adaptable to the subject(s) and may be performed during the actual exercise (depending on the protocol). Moreover, measurement of hydrocarbon gases is economical and requires limited facility.

Unfortunately, the production of hydrocarbon gases is considered a minor reaction pathway of lipid peroxide decomposition.
and may not reflect actual tissue oxidation [23, 103]. Hydrocarbon gas production and measurement may also be affected by bacteria as air pollutants (producing gas) and by oxygen concentration in vivo. Additionally, hydrocarbon gas production depends on the presence of metal ions to decompose lipid peroxides and so may not give an adequate index of the overall peroxidation process if such ions are only available in limited amounts [103]. Regardless of the above stated limitations, pentane and ethane continue to be used as a measure of biological lipid peroxidation. Although rigorous controls are required, the noninvasive nature of this detection technique will allow for its continued use.

2.10.2 Conjugated dienes

Attack of free radicals on the PUFAs of cell membranes causes the formation of hydroperoxides with conjugated dienes [104]. These conjugated diene structures which are formed during the peroxidation of PUFAs may be detected by their ability to absorb ultraviolet (UV) light in the wavelength range of 230-235 nm. This property of UV absorbance allows for measurement of an early stage in the peroxidation process and is useful in studies of pure lipids [28]. This simple method of diene-conjugate measurements is applied to lipid extracts from human body fluids, most commonly serum.

This method is less frequently used as it seems to have greater utilization when analyzing bulk lipids as opposed to biological materials. It has been reported that there are serious problems which can arise when used on human body fluids [103]. It has been described that the diene conjugates from human body fluids is produced by hydrogen abstraction from linoleic acid and reaction with the resulting
carbon-centered radicals with protein. The preferred reaction of carbon-centered radicals, however, is with oxygen, not with protein [28]. Furthermore, there is no universal agreement that there is conclusive evidence to support the idea that the conjugated dienes found in plasma is a product of oxidative stress. Few studies apply this method to their research efforts as great caution must be exercised in applying conjugated diene methods to biological systems and materials.

2.10.3 Malondialdehyde (MDA) and Thiobarbituric Acid Reactive Substances (TBARS)

One of the most commonly used assays for the detection and measurement of lipid peroxidation is the determination of TBARS by the thiobarbituric acid (TBA) test. TBA may be used as a test reagent to follow the progress and extent of lipid peroxidation in biological systems. Previous studies have shown that the amount of red product as noted by absorptiometry produced in the TBA test from aerobically incubated tissue was proportional to peroxide content and oxygen consumption during PUFA oxidation [33]. Although TBARS may be produced as a result of PUFA peroxidation, this substance is also associated with MDA. Since MDA reacts with TBA in acid to generate a red pigment having a visible absorption spectrum identical to that of PUFA-derived TBARS, it is generally felt that some of the measured TBARS may also represent MDA [33].

Malondialdehyde is one of several low molecular weight end products formed as a consequence of the decomposition of various lipid peroxidation products. Under appropriate conditions, MDA readily reacts with 2-thiobarbituric acid (TBA), generating a pink or red
chromagen that represents a MDA:TBA adduct in a 1:2 proportion. This chromagen may be measured by its fluorescence or its absorbance characteristics.

Some of the MDA detected in the TBA test is formed during the peroxidation process itself, but most is generated by decomposition of lipid peroxides during the acid-heating stage of the test, a process that is accelerated by transition metal ions in the TBA, acid and substances being tested. Thus, the TBA test does not measure MDA formed in the peroxidation system, therefore the term TBA-reactive substances (TBARS) is a much better term to use [105, 106].

The TBA test for the measurement of TBARS (MDA) is in common use and has stood the test of time for its usefulness. Many of the previously quoted studies have relied upon this test procedure to determine the presence of lipid peroxidation and asserts to its widespread use. It is a rather simple test to perform and requires limited technological resources. This test has fairly wide utility as it may be applied to defined membrane systems such as microsomes as well as body fluids and tissue extracts. It is also a relatively inexpensive test with high sensitivity and experimental ease of the test procedure. In addition, the TBA test does reveal the presence of TBARS in a reliable manner.

Determining the presence of TBARS as an indicator of lipid peroxidation is not without its limitations, however. Although simple to perform, the TBA test is a nonspecific assay. There may be other aldehydes other than MDA present in the sample with similar absorbance and/or fluorescence. A second difficulty is the fact that a variable amount of the MDA produced in the TBA test is formed during
the acid-heating phase of the assay, and consequently may give misleading results [9, 105]. It has also been shown that the presence of transition metals (particularly iron) invite artifactual oxidation of sample PUFAs during the testing procedure [33]. Lastly, the test may be affected by the presence of chain-breaking antioxidants in the sample. The greater the sample content of the antioxidant, the lower will be the TBA reactivity [28]. (Although this may be viewed as negative, this may also be one way of determining the effect of antioxidant use on peroxidation within the sample).

In spite of these limitations, the measurement of TBARS and MDA by the TBA test continues to remain a common fixture in the assessment of lipid peroxidation in the scientific community. Indeed, the value of the TBA test is that the peroxidation process beginning in the reaction mixture is effectively amplified in the assay itself, and in so doing, making it very sensitive [106]. In reviewing the literature it is readily apparent that this procedure is frequently employed. Although a nonspecific assay, with the institution of rigorous controls during the test procedure, many of the shortcomings will be diminished or nearly eliminated.

2.10.4 Electron Paramagnetic Resonance (EPR)

Each of the previously discussed techniques used for the detection of lipid peroxidation was an indirect measure of the formation of free radicals. This use of secondary indicators of free radical production is limited by the potential flaws in the procedures as well as assumptions that must be made when using an indirect measure to record any event.
Due to the high reactivity of free radicals, suitable techniques to detect their presence is a difficult task. Electron spin (paramagnetic) resonance (ESR or EPR) is a spectroscopic technique that detects the unpaired electron present in a free radical. As such, it is the only approach that can provide direct evidence for the presence of a free radical. In addition, the analysis of the ESR spectrum generally enables the determination of the identity of the free radical [107].

Spin trapping is a technique in which a short-lived, reactive free radical combines with a diamagnetic ("spin trap") molecule to form a more stable free radical ("radical adduct") which can be detected by electron spin resonance [107]. This technique involves the addition of a primary radical across the double bond of the diamagnetic compound to form an adduct more stable than the primary radical [108].

This technique is by far the most accurate way to determine the existence of free radical formation in the biological system being evaluated. As this is a direct measurement of the generation of free radicals, the requirement of assumptions utilizing indirect evidence is avoided. ESR represents the method with the most reliability and dependability.

In spite of the great promise of this technique, ESR is not without its own limitations. One of the major limitations is the availability and cost of this type of technology. The equipment needed for this type of analysis will only be found in the larger research facilities in those actively involved in this type of research. As such, the accessibility of this resource will be prohibitively restricted. The equipment is also very expensive to acquire and maintain further restricting is availability and usefulness. In addition, the sensitivity is
directly dependent on frequency (of spin resonance), and low-frequency instruments have been unable to achieve, as yet, the sensitivity needed to detect the low concentrations of radical adducts generated in vivo [108].

In addition, there inherent difficulties with the sample itself. The radical adduct must be stable long enough to not only survive the biological environment in which it is made, but also the time that is required to prepare the sample for analysis. The techniques used to obtain the sample requires an extraction technique relying on homogenization of the tissue. As a consequence, the physical destruction may itself lead to free radical formation in some manner (i.e., the release of iron and subsequent hydroxyl radical generation) [108]. Recent approaches have attempted to decrease this potential problem by examining biological fluids such as urine, bile, and blood (or plasma) directly for spin adducts [108].

Mason and Knecht have stated that in practice, both extraction of tissues and the examination of biological fluids should be used. They also noted that regardless of how the samples are obtained for ESR analysis, the relatively short lifetime of many radical adducts will preclude their detection in in vivo experiments. Additionally, they expressed the thought that ex vivo lipid peroxidation is clearly a concern in all in vivo experiments where sample handling and treatment occur and needs to be addressed no matter what technique is used to assess free radical formation [108].

In summary, although spin trapping is the most direct method for the detection of highly reactive free radicals in vivo and highly desirable, its major limitations are its high economy of use and
availability. This latter issue will preclude its use in many, if not most, research settings.

2.10.5 Serum Enzymes

Certain serum enzymes have been used as diagnostic markers of musculoskeletal injury, most notably, creatine kinase (CK) and lactate dehydrogenase (LDH). CK is found almost exclusively in myocardium, skeletal muscle, and brain. CK catalyzes the reversible transfer of energy-rich phosphate from creatine phosphate to adenosine diphosphate, thus forming adenosine triphosphate (ATP). The ATP formed is used mainly by the muscle during contraction. Thus, CK is an essential enzyme for the maintenance of energy in the muscle cell during activity involving muscle contraction, such as long-term or short-term exercise. The enzyme LDH catalyzes the reversible oxidation of lactate to pyruvate and is widely distributed in mammalian tissues, being rich in myocardium, kidney, liver, and muscle.

Unaccustomed or novel physical exercise involving varying types of muscle contractions has been shown to produce skeletal muscle damage. Each type of muscle contraction exerts a unique effect on skeletal muscle. Concentric and isometric muscular contractions are known to initiate increases in these enzymes, but to a much lesser extent than eccentric contractions. In other words, eccentric contractions result in a much higher increase than concentric contractions. It has also been documented that there are definitive gender differences between men and women. Namely that women exhibit lower serum enzyme activity at rest compared to men. This gender discrepancy is even more pronounced after exercise [109].
Kanter et al have attempted to suggest a correlation between serum enzyme elevations and exercise-induced lipid peroxidation [110]. Pre and post race serum MDA, CK, and LDH levels were studied in runners following an 80 km (50 mile) race. MDA, as noted above, is an indicator of lipid peroxidation. Nine male subjects were used with an average age of 47.4 years (range 35-60 years). These subjects had a mean maximum oxygen uptake of 48.2 ml/kg, and averaged 75 miles per week in training. Throughout the race, runners maintained a pace approximating 72% VO$_{2\text{max}}$. MDA concentration was measured by TBA assay. Serum was obtained one hour prior to the race and within two minutes of recovery following the race.

The postrace serum enzyme changes were as follows: CK 225%, CK-MB 171%, and LDH 108%. MDA rose by 77% at the conclusion of the race. All post race values were significantly greater than resting values.

These researchers stated that previous data from their laboratory indicated a high correlation between resting MDA and total CK and CK-MB. The resting data collected in this study served to confirm their earlier findings. In addition, the relationship established at rest persisted following exercise.

It was concluded in this study that post exercise serum enzyme elevations, universally accepted as a marker of tissue damage, correlate well and may be related to exercise-induced lipid peroxidation.

Although this study was well designed there are problems in assuming that this correlation will always be a valid one. There is
great variability in the activity of these enzymes, not only in the types of exercise sessions (mode, rate, intensity), but also among individuals (age, muscle mass, health status) and even within the same individual. Training status will have a profound effect on the analysis of these measures, as the less trained subjects will have the greater skeletal muscle damage, yet may not be subject to the most oxidative stress. The results gleaned from this study do indeed correlate serum enzyme levels with measures of lipid peroxidation. However, more data must be accumulated to state that these correlations are useful in other exercise settings and in a variety of subjects.

2.11 Possible Justification for the Use of Nutritional Antioxidants

Previous discussion has dealt with the fact that free radicals may be formed during the oxidative stress of exercise. The evidence supports the notion that as the utilization of oxygen increases, so does the flux through the mitochondrial electron transport system with resultant increased free radical production [15, 90, 93, 94]. In addition to the increased formation of free radicals there are changes which occur within the tissues to combat free radical attack. Protective mechanisms are present to ward off these harmful effects. For example, an acute bout of exercise has been shown to increase the antioxidant activities of certain enzymes, i.e., superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) in skeletal muscle, cardiac muscle, and the liver [15, 48, 111, 112]. Although it appears the tissues are able to adapt in an attempt to minimize free radical damage, the mechanism by which antioxidant enzymes can be activated within such a relatively short period of time during exercise is largely unknown [112].
As stated in the above paragraph, it has been demonstrated that acute exercise will induce such enzymes as SOD, CAT, and GPX. Similarly, chronic exercise will result in a similar induction of these enzymes. In particular, Mn-SOD and mitochondrial GPX show a greater induction in response to training in comparison to the other antioxidant enzymes [111, 112]. It seems that the training effects on catalase and Cu/Zn-SOD are less prominent when compared to the aforementioned enzymes [112]. Interestingly, skeletal muscle has one of the lowest antioxidant enzyme levels in the body, but oxygen flux may increase 10 to 20-fold during strenuous activity. The large increase in the production of free radical species may act as a stimulus for an increase in antioxidant enzyme synthesis, either directly or indirectly [112]. It has been demonstrated that the maximal activities of several antioxidant enzymes correlate with aerobic capacity among tissues [15]. In other words, tissues with high oxygen consumption, such as heart, brain, and liver, have relatively high antioxidant levels. Conversely, those tissues with relatively lower metabolism, such as skeletal muscle, have comparatively low levels of antioxidant enzymes. This observation relates well to the impression that tissues with high aerobic activity require greater protection from oxygen-derived radicals. This protection could be provided in the form of enhanced activity of antioxidant enzymes. These enzymes, however, may not be able to meet the needs of intense training and nutritional antioxidants may be necessary to combat free radical attack [45, 47, 49].
2.12 Exercise Enhances Antioxidant Enzymes

Various investigations have shown that antioxidant enzymes may be enhanced by exercise. A frequently studied "enzyme" is glutathione (GSH) which may be readily studied by using red blood cells. Erythrocytes utilize glutathione to prevent the oxidation of hemoglobin to methemoglobin and therefore may be studied for its antioxidant effects and concentrations. In an analysis of blood antioxidant status, Duthie et al studied seven trained runners before and up to 120 hours after completing a half-marathon (13.1 miles) [113]. During this investigation he monitored blood levels of conjugated dienes, TBARS, erythrocyte GSH, and creatine kinase (CK) activity. Skeletal muscle damage was indicated by a significant increase in CK following the race. However, there was no evidence of lipid peroxidation as there was no concomitant increase in TBARS or conjugated dienes. There were noted significant changes which occurred in erythrocyte GSH. There was a decrease in GSH within the erythrocytes immediately following the race allowing increased susceptibility of these cells to lipid peroxidation. These results indicated that there were significant changes in the GSH concentrations, i.e., antioxidant status, in the erythrocyte after the exercise. This effect was noted even when there was no elevation of plasma indices of lipid peroxidation indicating that blood antioxidant status is subject to change as a reflection of physical activity.

A different study using erythrocytes was performed with glutathione reductase activity (GR) being examined [111]. This study evaluated GR functioning utilizing sedentary males performing thirty (30) minutes of exercise on a bicycle ergometer at 75% VO2 max. (It
may be recalled that the function of GR is to restore GSH levels toward normal by oxidizing GSSG. As GSH levels decrease, GR should increase). These researchers found that the bout of acute exercise resulted in an increase in GR activity in an effort to maintain levels of GSH. Presumably, this increase in the free radical scavenging system is an attempt to maintain levels of reduced glutathione (GSH).

The above study evaluated the effects of an acute bout of aerobic exercise on antioxidant enzyme activity. A subsequent study performed by the same investigator evaluated the effects of 10 weeks of aerobic training as opposed to an acute episode [112]. In this study, GR activity within erythrocytes were measured as well as erythrocyte catalase activity. Similar to the previous study, the results of this study provided evidence that aerobic activity increased antioxidant abilities of the erythrocytes.

Robertson et al examined blood antioxidants in twenty runners and six sedentary individuals [116]. The runners were subsequently divided into two groups: low-training runners (16-43 km/wk) and high-training runners (80-147 km/wk). Plasma CK activity was used as an indicator of muscle damage and had a significant correlation to weekly mileage. Erythrocyte activities of the antioxidant enzymes, glutathione peroxidase and catalase, were significantly and positively correlated with the weekly training mileage. Total erythrocyte glutathione content was higher in the two training groups and was accounted for by an increase in reduced glutathione content. These results indicated that there is an increase in blood antioxidant defense mechanisms associated with endurance training.
Ji et al examined the effects of exercise on antioxidant enzymes in skeletal muscle [48]. In this investigation, glutathione status and antioxidant enzymes in various types of rat skeletal muscle were studied after an acute bout of exercise at different intensities. This study was undertaken with the knowledge that maximal activities of several antioxidant enzymes, e.g., SOD and CAT, correlate with aerobic capacity among various tissues such as muscle, red blood cells, lung and brain [15]. The results of this study demonstrated that an acute bout of exercise significantly increased activities of certain antioxidant enzymes such as GPX, GR, and CAT. These findings support the data from the previously mentioned research that activation of antioxidant enzymes may be observed after exercise, i.e., exercise enhances antioxidant enzymes.

As is usually the case in the scientific arena, not all research agrees with the above data. A study utilizing submaximal cycling exercise in a repeated fashion on moderately trained men produced no rise in plasma GSSG. Presumably with free radical production, the level of GSSG should increase in response to reduction of hydrogen peroxide to water. In this study, however, this was not the case. These investigators obtained no evidence that repeated bouts of exercise induced sufficient oxidant stress to result in cumulative or persistent effects on the blood glutathione redox status [114].

Other studies have given conflicting, and at times, confusing results. A comparison of three separate studies bears this out. For example, one study performed by Ji et al provided results which demonstrated no change in GSSG and an increase in GSH which contradicted a study done by Gohil et al which revealed a decrease in
GSH and an increase in GSSG [118, 119]. Confounding this even further is a report that reported no change in GSH and an increase in GSSG [120].

The findings from these studies are not easily resolved. There was no standardized protocols used in them. The exercise regimens were different as were the method of testing and the fitness levels of the participants. Additionally, the nutritional status of the subjects were not identified. It is therefore not possible to ascertain the definitive rationale for the dissimilarity.

2.13 Nutritional Antioxidants can augment endogenous defenses

Increases in plasma and tissue antioxidant concentrations and antioxidant enzyme activities in trained athletes compared with sedentary individuals indicate adaptive upregulation of the antioxidant defense system. This is likely to be a response to enhanced, persistent oxidative loads arising from sustained exercise [118]. However, such adaptations may be insufficient to completely protect an individual who trains extensively. This has been noted in various studies some of which have already been alluded to in previous discussion. For example, the study comparing blood glutathione concentrations and creatine kinase activities in athletes who consistently train (run) 80-147 km/wk [116].

There has been a considerable amount of investigation which has been undertaken to evaluate the effects of nutritional antioxidants on exercise-induced oxidative stress. Results from these studies have provided encouragement for a benefit in reduction of the adverse effects of tissue oxidation [10, 96, 119]. There appears to be a reduction of the detrimental repercussions of oxidative stress which
occurs with exercise. This will be detailed under antioxidant supplementation and exercise-induced lipid peroxidation.

In addition, most of the reports in the literature that have evaluated the increase in antioxidant enzyme activities following exercise have reported enhancement in the order of 15-50% [121]. Oxidative enzyme activity, on the other hand, usually rises to a much greater magnitude as a result of a training effect. As a consequence, there is an overall decrease in the ratio of antioxidant protection as compared to oxidative capacity [108, 120, 121]. These findings would suggest that an individual actively involved in an aerobic training program may not possess adequate antioxidant defenses to counteract the oxidative stress that occurs as part of the outcome of exercise training. This would indeed lend credibility to the implication that individuals who regularly engage in physical activity would derive benefit from supplementation of antioxidants.

Nutritional antioxidants are present to bolster the antioxidant enzyme activities, but their quantity may be inadequate to provide the greatest benefit. It is this premise that allows justification to be made to foster the use of antioxidant supplements in a training program. This will be detailed in section 2.15 of this dissertation entitled, Antioxidant Supplementation and Exercise. It is in this section that the results of studies which have evaluated the effects of supplementation will be presented.
2.14 Synergistic Interrelationship between Vitamin C and Vitamin E

Vitamin C, or ascorbic acid (ascorbate) and vitamin E (tocopherols) are both noted for many functions. Relevant to this discussion are their roles in antioxidant ability. Vitamin C is water-soluble and is considered to be the most important antioxidant in extracellular fluids [31]. As noted from earlier discussion, vitamin C has been shown to scavenge superoxide, hydrogen peroxide, and the hydroxyl radical. Vitamin E is fat-soluble and is able to function as an antioxidant due to its integral relationship with cellular membranes. As an \textit{in vivo} antioxidant, vitamin E is able to protect tissue lipids from free radical attack and peroxidative damage.

In addition to their separate and distinct roles in antioxidant function, there does exist a synergistic interrelationship between vitamin C and vitamin E. As noted in a previous section, vitamin C may \textit{directly} quench free radical species by reacting with superoxide anion and/or stabilizing the hydroxyl radical. However, vitamin C may also act \textit{indirectly} as an antioxidant via regeneration of reduced vitamin E, renewing its ability to function as an antioxidant. This regeneration of active, membrane-bound, chain-breaking vitamin E enables this vitamin to protect the cellular membranes from lipid peroxidation (Figure 12). Vitamin E is able to terminate chain-propagation reactions by reacting with the peroxyl radical and preventing further abstraction of hydrogen from the fatty acids within the membrane. Vitamin E, in the reduced state, is able to provide a hydrogen for the reduction of the lipid peroxy radical. However, the oxidized vitamin E must be regenerated to maintain its functional
Figure 12. Antioxidant activities and recycling of vitamin C and E. GSSG is oxidized glutathione, GSH is reduced glutathione, and LOO* is a lipid peroxyl radical. Ascorbic acid is oxidized to dehydroascorbic acid, which can be regenerated to the reduced or active form by glutathione. Alpha-tocopherol is oxidized to a tocopheroxyl radical in reacting with a free radical and then may be regenerated to the active, reduced form by ascorbate.
activity. In vitro studies have provided evidence that vitamin C reduces the tocopherol radical and subsequently restores the radical-scavenging ability of vitamin E [1, 127]. The tocopherol radical that forms in membranes is thought to react with the ascorbate to yield tocopherol and the ascorbyl radical, the result of which is to maintain radical scavenging potential within the membrane by regenerating tocopherol and to transfer the oxidative challenge to the aqueous phase [1, 69, 72]. This regeneration of oxidized vitamin E is thought to require not only vitamin C, but NADPH and reduced glutathione as well.

Although such a synergistic activity has been shown using model systems, direct and unambiguous evidence for its occurrence in biological systems has yet to be definitively presented [1, 69].

2.15 Potential Ergogenic Effects of Antioxidant Supplements

Athletes at all levels of competition are continuously searching for any means that will give them a competitive advantage over the opposition. In spite of the fact that the key to athletic success involves proper biochemical, physiologic, and psychologic training, the search for an ergogenic enhancement continues undaunted. It is thought that performance may be enhanced by improving either energy efficiency, energy control, and/or energy production during exercise through the use of various ergogenic aids.

Ergogenic aids may be divided into several general categories. Such things as improvements in shoes, or orthotics would constitute biomechanical aids. Pharmacologic aids includes such things as anabolic steroids and a whole host of available drugs. Physiologic aids
could involve blood doping to cause an increase in red blood cell mass to improve oxygen delivery. Hypnosis could be used as a psychological aid. In particular and relevant to the overall discussion in this document is the use of antioxidants as a nutritional ergogenic aid. Dietary intake plays a major role in performance and it is in this regard that athletes have looked to vitamins and minerals in order to presumably provide optimal and efficient use of available energy. Athletes presume that there might not be adequate quantities of certain nutrients in their normal diet and that increased amounts may be beneficial to their endeavors.

It is a well-known fact that exercise performance will be impaired as a result of a micronutrient deficiency. Decreases in performance may be documented when there is a reduced dietary intake or serum levels (or both) of one or more vitamins and/or minerals. A prominent and frequently occurring example of this is iron deficiency anemia.

As stated earlier in this review, the generation of free radicals is a normal occurrence. These free radicals, as a consequence of their nonspecific reactivity and harmful effects, may damage and/or alter cellular membranes and molecular structures. Due to the fact that increased physical activity obligatorily increases oxygen consumption, generation of free radicals will be increased as well. Increasing exercise intensity has been suggested to be associated with increased production of free radicals [123]. A lowered antioxidant status has also been shown in humans with intense physical activity [124]. In addition, the production of fatigue during exercise has been noted to be likewise associated with the formation of free radicals [104]. It is
with this in mind that a rationale for supplementation of antioxidants may be proposed. In essence, antioxidants could serve as an ergogenic aid.

The potential beneficial effects of the use of antioxidants is not a universally held belief however. Although there have been many studies and investigations, much controversy still exists. For example, it has been stated that "There is little if any evidence that nutritional supplements have an effect on performance or muscle mass in athletes consuming a balanced diet; some of these products have the potential to induce adverse effects, however" [125]. In direct opposition to this statement is the following: "A final conclusion from the brief overview of research on nutritional ergogenic aids is that there is no question that dietary manipulation or supplements can improve human exercise performance in certain settings. A prevailing opinion that supplementation of athletes with nutrients is useless, harmful, or quackery is rapidly melting under the heat of scientific findings" [126].

The following section of this review will specifically deal with antioxidant supplementation. The discussion will focus on arguments for and against the use of vitamin E, vitamin C, antioxidant mixtures (which includes beta-carotene), and antioxidant compounds as a means to enhance exercise performance.

2.16 Antioxidant Supplementation and Exercise

Several studies which have frequently been cited in the literature concerning free-radicals, exercise, and antioxidant supplementation will be reviewed. Although there is a large body of literature available to the interested reader, it is impossible to discuss each and every
study. Therefore, this review will be limited to the better studies that have been done (as there are many that are plagued by poor design). Most of the studies that have been done have used less than ten subjects. An attempt has been made to discuss studies which have larger study populations, where available. An attempt has also been made to review the most frequently quoted studies used to support a particular point or prove a specific observation.

2.16.1 Vitamin C Supplementation

Frei undertook an investigation of vitamin C, ascorbic acid, and provided evidence that it has the ability to protect lipids against oxidative damage [127]. This researcher exposed human plasma to different oxidative challenges, such as cigarette smoke, activated neutrophils, and chemically generated peroxyl radicals, and followed the temporal consumption of endogenous antioxidants in relation to the initiation of oxidative damage. Under each type of oxidizing condition, the ascorbic acid completely protected lipids in the plasma against any detectable peroxidative damage. This study reported that vitamin C is the most effective antioxidant in the plasma.

One of the notable elements of this study was the manner in which the oxidative damage was assessed. The very sensitive and selective high-performance liquid chromatography (HPLC) assay was utilized. This method measures lipid hydroperoxides directly thus allowing for an accurate determination of by-products of oxidative damage. Many other studies have used indirect indices of lipid peroxidation such as diene conjugation or thiobarbituric acid reactive substances which have their limitations and were discussed in an earlier section (section 2.10).
Frei concluded that his data proved that ascorbic acid was able to function as a very effective antioxidant and afford protection to plasma lipids against detectable peroxidative damage induced by different types of oxidants. From the data presented in this study, it would seem reasonable to consider vitamin C as a supplement to protect against oxidative stress.

Although this study did provide rather solid evidence for the role of vitamin C as a protector against peroxidative damage, this data may not be readily applicable to the working muscle and body. This research was confined to in vitro laboratory conditions and was not performed within a true physiological state. Equating artificially induced stress in a test-tube does not always relate to the aerobically exercising individual. There are many variables that are only present in the human and may not be reproduced experimentally as done here. In particular, it is difficult to determine an appropriate dosage required by the exercising person to achieve the antioxidant benefits suggested by this research.

In spite of this obvious limitation, these data do provide evidence that ascorbic acid does protect lipids against oxidative damage and certainly is very encouraging and suggestive as a rationale for its supplementation.

Keith and Driskell performed a study evaluating lung function and treadmill performance and ascorbic acid supplements [128]. This study evaluated twelve cigarette smokers (10 pack-years or more) and ten nonsmokers (never smoked), all males between ages 25 and 38 years. Both of these groups were untrained and did not participate in any regular exercise program. This was a double-blind crossover study.
The study consisted of three periods. The first period involved three weeks of either supplementation of vitamin C at a dose of 300 mg per day or placebo. Each of the exercise parameters were measured weekly. After 3 weeks the subjects were taken off the tablets for 4 weeks. During this four week interval, no exercise tests were performed. This wash-out period was chosen because it has been reported that ascorbic acid has a half-life of 15 days. In fact, this group of researchers performed a preliminary study to verify this and it was found that vitamin C levels did return to initial values in supplemented subjects in four weeks after discontinuation. During the third period (3 weeks) the subjects received the tablets not given during the first period and measurements were performed weekly.

One of the particularly useful bits of information which was performed in this investigation (and frequently omitted in other studies) was the actual measurement of plasma levels of vitamin C. It was reported that plasma levels of vitamin C rose significantly as compared to those with and without supplementation, and clearly substantiate that increased levels were achieved. The data obtained in the study indicated that ascorbic acid supplements appeared to have little effect on lung function and physical performance.

One of the faults of the study is that there is "significant" overlap within the standard deviations in the actual plasma values. With such a small number of subjects, 3-4 subjects at any one time could have been deviating the levels and impairing the results regardless of whether it was the supplemented group or the placebo group.
Another flaw of this design concerns the dosage of 300 mg per day of ascorbic acid which was probably too small to show any kind of effect (either positive or negative). It has been shown that a dose of 200 mg/day does not fully saturate the plasma. The plasma is approximately 80% saturated at this dose, furthermore, the plasma saturates at 1000 mg per day [129]. It is also important to bear in mind that the data for plasma and cells are based on administration of pure vitamin C alone at least 1.5 hours before meals. In this study, the timing of administration was not specified, nor was there any indication of the timing of meals.

Moreover, the subjects in this investigation performed treadmill exercise at 50% of their VO_{max} as estimated by heart rate [128]. This may not have provided enough physiological stress to enable the observance of any difference in physical ability. The treadmill workload and ventilation measurements were performed during the last minute of the 5 minute exercise period. This method did not allow for any observance for changes during the initial phases of the exercise and involved only five minutes of exercise. Although it has been stated that oxygen radicals are formed even at rest, the normal body's defenses are likely to cope with this quantity. In order to reveal that an antioxidant effect was enhanced, the exercise bout should be more stressful and longer than the normal amount that might occur at rest. An increase of oxygen-radical generation with an appropriate stimulus is needed to demonstrate an effect. It is doubtful that any effects could be seen with this "less-than-stressful" exercise bout.
These investigators also measured postexercise lactic acid values [128]. The blood lactic acid levels of both the smokers and nonsmokers decreased gradually across the 3 week period while on the ascorbic acid supplementation. There was great individual variation however. Blood lactate levels did decrease as supplementation continued in both groups suggesting either a beneficial effect and/or a training effect.

One last point from this study was noted in the data but not emphasized by these researchers. Very brief mention was made of the fact that both groups tended to have lower postexercise systolic blood pressure readings, higher VO_{\text{max}}, and lower postexercise blood lactic acid values during the ascorbic acid treatment than during the placebo. Emphasis was probably avoided here due to the fact that none of the values reached statistical significance.

Research performed by Alessio and Goldfarb attempted to describe how vitamin C supplementation affects oxidative stress balance by assessing a prooxidant biomarker, thiobarbituric acid reactive substances (TBARS), and a total antioxidant activity biomarker, oxygen radical absorbance capacity (ORAC), before and after 30 minutes of exercise at 80% VO_{\text{max}} [94].

In this study, nine male subjects were used. All of them were stated to be young (mean age was 33 years ± 2.6 years), fit (VO_{\text{max}} was 56.24 ± 2.5 ml/kg/min) and highly motivated. Each subject participated in all three phases of the investigation in a randomized order: submaximal exercise with placebo for 1 day, one day of 1 g vitamin C supplement, and two weeks of 1 g/day vitamin C supplement. Supplements were taken in two doses of 500 mg each with
meals. There was a two week period of no supplements between each condition.

Following an acute bout of exercise at 80% of VO$_{2\text{max}}$, TBARS levels averaged 12.6% above resting levels when the subjects had been supplemented with vitamin C for 1 day, 33% above resting levels when supplemented for 2 weeks, and 46% above resting levels when they received a placebo. These changes in TBARS before and after exercise however reached significance only when subjects were not supplemented.

ORAC, a biomarker of total antioxidant activity in plasma, tended to rise 12.6%, 4.9%, and 5.7% above resting levels after submaximal exercise for the placebo treatment, for 1 day vitamin C supplementation, and for 2 weeks of vitamin C supplementation, respectively. Similar to TBARS, these changes in ORAC levels following exercise were not significant regardless of vitamin C supplementation. Overall, the prooxidant:antioxidant balance, as indicated by the TBARS:ORAC ratio, was tilted most toward oxidative stress when the subjects had no vitamin C supplements (32.5%). This reached significance only when compared to the one day supplementation. Two weeks of vitamin C supplementation resulted in a 25.5% change in TBARS:ORAC after exercise, but failed to reach statistical significance than either of the other two conditions following exercise.

This study did show that only under exercise-induced oxidative stress were the effects of vitamin C supplementation noticeable. Intuitively, this makes sense, as oxidative stress under resting conditions would be expected to be minimal (while during exercise
production of free radicals is increased). This study did reveal (as expected) that the prooxidant:antioxidant balance was approximately the same regardless of supplementation at rest.

One of the potential flaws of this study design is the manner in which oxidative by-products were assessed. TBARS was used for this data collection. This assay does have an inherent problem of lacking specificity when applied to human plasma. There are laboratory adaptations that may be undertaken to avoid some of these obstacles. Unfortunately, it is unknown if some of these adaptations were used to obtain any of these values. Furthermore, while the work of Alessio and Goldfarb does suggest that vitamin C supplementation reduces exercise-induced oxidative stress, it does not provide insight into any possible ergogenic benefit. This was a short-term study that did not address performance. Hence, how a vitamin C induced reduction in oxidative stress relates to enhancement of exercise performance remains unanswered.

Studies by Jakeman and Maxwell highlight the possible importance of changes in antioxidant status and supplementation during eccentric exercise. Jakeman and Maxwell investigated the effects of antioxidant vitamin supplementation upon muscle contractile function following eccentric exercise in a double-blind fashion [130]. This study was undertaken to determine whether dietary supplementation of antioxidant vitamins, namely vitamin C and vitamin E, could attenuate the loss of contractile function following eccentric exercise.

This study utilized a box-stepping protocol to induce eccentric muscle stress/damage. The muscles used were the triceps surae,
namely the gastrocnemius and the soleus, which are known for their high oxidative capacity and are predominantly slow muscles. These muscles are more resistant to fatigue as compared to fast muscle fibers and therefore less vulnerable to free radical injury and oxidative stress. Twenty-four physically active young subjects (mean age 19.6 years) ingested either placebo, vitamin E (400 mg), or vitamin C (400 mg) for 21 days prior to and for 7 days after performing one hour of box-stepping exercise. Contractile function of the triceps surae was assessed by the measurement of maximal voluntary contraction (MVC) and the ratio of the force generated at 20 Hz and 50 Hz tetanic stimulation before and after eccentric exercise and for 7 days during recovery. Vitamin C (400 mg/day for 21 days) promoted less low-frequency fatigue (indicative of exercise-induced muscle damage) than did vitamin E or placebo [130]. This investigation also revealed that recovery of maximal voluntary contraction during a 24 hour post-exercise period was also greater in vitamin C supplemented subjects.

Following the eccentric exercise, MVC decreased to 75% of the preexercise values and the 20/50 Hz ratio of tetanic tension from 0.76 to 0.49. Compared to the placebo group no significant changes in MVC were observed immediately post-exercise for either vitamin C or E, though recovery of MVC in the first 24 hours post-exercise was greatest in the group supplemented with vitamin C. The decrease in 20/50 Hz ratio of tetanic tension was significantly less (P < 0.05) post-exercise and in the initial phase of recovery in subjects supplemented with vitamin C, but not with vitamin E. These data suggested that prior vitamin C supplementation may exert a protective effect against eccentric exercise-induced muscle damage.
The authors assumed that the use of these muscles in this fashion resulted in oxidative stress yet did not provide any measures of this phenomenon either by direct or indirect measure of free radical generation or ROS production. Increased oxidative stress was not documented in the data.

The omission of any biomarker of oxidative stress in the above study was addressed in a very similar study performed by the same researchers [131]. This study also utilized healthy subjects (24) undertaking one hour of box-stepping exercise. Similar doses were used as well as placebo as in the previous study (i.e., vitamin C 400 mg/d, vitamin E 400 mg/d). This study reported no significant changes in plasma malondialdehyde (MDA), a frequently used biomarker of oxidative damage. The speculation would be similar to the statements in the previous paragraph, and in fact, verify the suggestion that the mode of exercise used in the study did not cause significant free radical production or oxidative stress to be noted by the MDA. Alternatively it could be argued that an increase in antioxidant status (as found in this study [131]) was sufficient to quench any oxidants that were produced.

In this second study by Jakeman and Maxwell, although the muscles did respond to the eccentric exercise with elevations of creatine kinase as well as decreased MVC and the 20/50 Hz ratio of tetanic tension, there is nothing in the data to verify that this was in fact due to oxidative injury or simply a function of eccentric exercise-induced muscle damage. Another factor related to the muscle was the fact that these subjects were “physically active” and “fully habituated to the experimental procedures”. Other than stating the mean
maximal oxygen uptake of the subjects, no notation is made as to the amount or kind of training (if any) the subjects were accustomed to performing. There was no specification of the meaning of those terms. Various fitness routines might tend to favor some subjects over some of the others in the performance and response to the exercise and the results of the study.

A study performed by Peters, et al evaluated long distance runners who ingested vitamin C 600 mg/d for 21 days prior to an ultramarathon (90 km) race [132]. They reported that there were fewer cases of upper respiratory infections in runners who ingested vitamin C than runners who did not ingest the supplement. From their data, it is not possible to determine if these apparent benefits were due to the antioxidant properties of the ascorbic acid or some other factor. Although it would seem that the distance of this ultramarathon would impart oxidative stress, the increased susceptibility to infection is a very nebulous situation and a poor marker of oxidative stress. One of the confounding factors not discussed or addressed in this study is the amount of exposure to illness encountered by these runners during their daily living. Those runners with the most exposure would certainly be the most likely to become infected. Moreover, Peters did not measure any biomarkers of lipid peroxidation or oxidative stress to verify that this was present. Certainly such an extreme distance of 90 km is sufficient to result in oxidative stress, yet this was not investigated. Nor was there any clear data to demonstrate that the plasma levels were changed by the supplementation that would suggest a beneficial increase. In addition, there was no direct evaluation of the effects of the vitamin C supplementation on exercise performance.
A recent double-blind study compared the effects of a low dose (500 mg) of vitamin C to a high dose (2000 mg) on muscular strength, endurance and maximum oxygen consumption [133]. Both acute (four hours prior to testing) and chronic (supplementation for one week) modes of administration for each dose were tested. Determinations were performed on twenty-four subjects for muscular strength (quadriceps and pectoral muscles), muscular endurance, and VO\textsubscript{2max}. Significant improvements were noted in quadriceps strength. However, there was no effect on quadriceps endurance or pectoral strength or endurance with the acute dosing of vitamin C 500 mg. In addition, there was a significantly reduced VO\textsubscript{2max}. Acute dosing of 2000 mg produced no significant changes in oxygen consumption, muscular strength or endurance. Further testing was performed following chronic supplementation of 500 mg for seven days. This supplement regimen resulted in significantly increased quadriceps and pectoral muscle strength, reduced muscular endurance (quadriceps and pectoral) and significantly reduced VO\textsubscript{2max}. The only noticeable effect with 2000 mg ingestion for seven days was a significant reduction in VO\textsubscript{2max}. This study resulted in several contradictory findings which makes interpretation difficult. Also, there was no discussion of any oxidative biomarkers in this data which would have clarified the known role of the antioxidant effects of vitamin C on these parameters. Although acute dosing may have resulted in some effect, there are many factors that affect vitamin C absorption. Unfortunately, the measurement of the plasma response to the
vitamin C administration was not reported. Furthermore, it has not been established whether chronic supplementation for only seven days is a sufficiently long enough period of time for chronic effects on exercise to be noted.

In conclusion, there is no doubt that vitamin C has antioxidant function. There are many studies that hint at the benefits of its use as an ergogenic aid. However, further detailed research is necessary employing double-blind controls with randomized crossover designs and large numbers of subjects and large doses of ascorbate to more fully evaluate the ergogenic effects of vitamin C.

2.16.2 Vitamin E Supplementation

Brown, et al evaluated the effects of vitamin E supplementation on indexes of lipid peroxidation [134]. They studied two groups of fifty males each, one group was comprised of smokers (> 10 years, > 15 cigarettes/d) and another group was made up of nonsmokers (it was not reported if any were former smokers). All were reportedly healthy factory workers who were on no medication or supplements. There were four treatment groups (smokers vs nonsmokers and placebo vs vitamin E supplementation). For a ten week period, each subject was instructed to ingest either a placebo or 280 mg alpha-tocopherol immediately after breakfast. Activity levels were not specified during the trial. Blood samples were drawn immediately before supplementation and then 10 weeks later after an overnight fast.

The results of the study were as follows: (1) plasma and erythrocyte vitamin E concentration did not significantly differ between smokers and nonsmokers at week 0 and increased equally as the study progressed. These values remained unchanged in the
smokers and nonsmokers receiving the placebo. (2) Before supplementation the susceptibility of the erythrocytes to hydrogen peroxide-induced peroxidation was markedly greater in smokers than in nonsmokers. (The susceptibility of erythrocytes to hydrogen peroxide-stimulated peroxidation in vitro was used as a functional measure of antioxidant status). This difference was abolished after vitamin E supplementation, which reduced erythrocyte peroxidation in both smokers and nonsmokers alike. (3) Plasma concentrations of lipid peroxides, TBARS, and conjugated dienes were originally higher in smokers than nonsmokers, but this difference was abolished after supplementation with vitamin E. All of the above support the claim that supplementation with vitamin E suppresses lipid peroxidation.

A noteworthy aspect of this study is the fact that two parameters of lipid peroxidation, conjugated dienes and TBARS, were assessed. Both of these measurements are indirect biomarkers of lipid peroxidation. Due to the imperfections of these biomarker measurements, the use of a second one will serve to confirm the results of the other enhancing the credibility of the data.

There are however, several shortcomings in this particular study. All of the subjects were over forty-five years of age. Aging is known to increase susceptibility to free radical attack and decrease antioxidant capacity. As these subjects were all middle-aged, this may have created a tendency for an increased lipid peroxidation due to the aging process. Secondly, there was no mention of compliance levels or dietary intakes of the subjects. Although the data were consistent, there would be wide variability within the data if there were compliance problems or various dietary practices. Additionally, the
investigators stated that the subjects were factory workers, but provided no indication of the exact physical characteristics or demands of the occupational requirements. It is conceivable that the indicators of peroxidation could be elevated due to heavy physical labor as opposed to someone with a more sedentary type of position. Finally, no mention was made of the actual activity levels of the subjects either before or during the period of investigation. This hampers the application of this data to the physically active individual and exercise performance. In spite of these limitations, it would appear that vitamin E may have some utility in the reduction of oxidative stress.

Sumida, et al, undertook an evaluation of the effects of exercise on lipid peroxidation as a function of vitamin E supplementation [135]. In addition, whether membrane damage resulting from lipid peroxidation was examined from the standpoint of leakage of enzymes from tissues into blood.

Twenty-one healthy males (mean age 20.3 ± 0.3 years) participated in this trial. Each ingested 300 mg/day of vitamin E for four weeks. Plasma levels of vitamin E were assayed, and found to increase from 9.6 ± 0.6 to 22.3 ± 1.1 μg/ml during the four week period. Each subject performed a control trial and a vitamin E trial before and after the four week vitamin E supplementation period. It is not clear from the article what was meant by this type of study design. It appears that all of the subjects ingested vitamin E. These same subjects underwent testing prior to vitamin E supplementation followed by repeat testing after four weeks of vitamin E. Likewise, it appeared that the subjects served as their own controls. The subjects
performed an incremental exercise test to exhaustion on a Monark bicycle ergometer. Blood samples were taken before the exercise, immediately after exhaustion, and at one and three hours into the recovery period. The lipid peroxide content was estimated by measuring malondialdehyde (MDA).

Significant differences were not found in maximal oxygen consumption, maximal heart rate, or exercise time to exhaustion between the control and the vitamin E trials. MDA concentrations in the vitamin E trial were significantly lower than those in the control trial (3.8 ± 0.2 nmol/ml vs 4.4 ± 0.2 nmole/ml at pre-exercise). MDA concentrations did not change from pre-exercise, or at 1, or 3 hours recovery. A slight but significant increase in MDA concentration (4.5 ± 0.2) was observed immediately after the control exercise.

Although this investigation showed a significant decrease in MDA, indicating a beneficial effect in combating oxidative stress due to exhaustive exercise, there was no significant prolongation of time to exhaustion. One of the factors that may have contributed to this outcome is the fact that the subjects began with lower levels of MDA prior to the ingestion of vitamin E. This may have been due to an effect of the fitness level of the individuals within this group. In this study, it was stated that “we used sedentary or moderately trained men”. This was the only mention of any hint of fitness levels in the subjects. Therefore, the observed differences with respect to MDA may be due to differences in the fitness level of the particular subject and/or the absolute intensity of the exercise bout. Nonetheless, their results indicate that lipid peroxidation occurs following a bout of exhaustive exercise in young males.
On the other hand, MDA levels in the serum did not increase immediately after the vitamin E exercise despite the fact that the same maximal exercise intensity was achieved in the vitamin E and the control exercise group. Rather, the MDA levels were decreased significantly immediately post-exercise. The speculative conclusion would be that the vitamin E supplementation inhibited lipid peroxidation following the exhaustive exercise.

Lastly, this study was not a double blinded design. Just because VO$_{max}$ remains the same does not mean that performance will as well. However, this would not seem to tarnish the results as the exercise parameters were virtually identical. In addition, there could be no placebo type of effect that would be detectable by plasma or serum analysis. Therefore, the data presented in this investigation would suggest that vitamin E is effective for the inhibition of oxidative membrane damage seen immediately following exhaustive exercise.

Another group of investigators evaluated the effects of antioxidant vitamins (E or C) on lipid peroxidation in subjects exposed to extreme endurance stress [136]. The intent of this inquiry was to establish whether extreme endurance (physiological) stress of trained athletes can influence lipid peroxidation and muscle enzymes. A randomized and placebo-controlled study was performed on 24 trained long-distance runners who were supplemented with vitamin E 400 I.U./d and vitamin C 200 mg/d for 4.5 weeks prior to a marathon race. The serum concentrations of retinol, ascorbic acid, β-carotene, alpha-tocopherol, MDA, uric acid, glutathione peroxidase, and catalase were measured 4.5 weeks before, immediately before,
immediately after and 24 hours after completion of the race. In addition, measurements of creatine kinase were taken before and after the race. Since the increase of CK serum concentration is remarkably lower in the supplemented group compared with the placebo group \((P < 0.01)\) immediately after and 24 hours after the marathon, these investigators concluded that endurance training coupled with antioxidant vitamin supplementation reduces blood CK increase under exercise stress. It was also discovered that the levels of TBARS in the serum decreased for both the supplemented group and the placebo group. The serum levels of ascorbate and tocopherol were both increased in the supplemented group as expected, however there was no concomitant increase in the glutathione peroxidase or catalase. This study is certainly suggestive of some benefit of the use of combining antioxidants, however it cannot be established if the results were due to either of the vitamins working alone or in summation. Also, the authors did not make a powerful argument to attest to their usefulness in exercise performance.

Finally, there is one other study that provides very encouraging evidence in the support of the use of vitamin E to enhance exercise performance [137]. Supplementation of vitamin E 400 mg/d to mountain climbers at altitudes above 5000 meters for ten weeks completely prevented the loss in anaerobic threshold seen in unsupplemented control subjects. Breath pentane exhalation increased 104% in control subjects, but decreased 3.0% in supplemented subjects. The conclusion reached was that vitamin E supplementation had a beneficial effect on physical performance and tissue protection at high altitude.
In contrast to the studies discussed above, there have been a variety of studies that have shown no significant differences in performance after supplementation with vitamin E [138]. Generally these studies have been well controlled and are almost as numerous as those studies that have reported a benefit with the use of vitamin E. Unfortunately, many of the studies that have shown a benefit have suffered from poor design or have employed different dosing regimens and different exercise intensities. Any or all of these differences in research design may account for the variations in the data. Further research is definitely needed in this area.

In summary, vitamin E is known to be involved in many processes within the human body and is an essential micronutrient. There is accumulating evidence that a reduction in oxidative stress from physical activity may be possible with vitamin E supplementation. However, there are very few studies on humans that have shown any benefit in well controlled study designs. The information from altitude studies suggest the use of vitamin E supplementation. Further research is necessary with well done controls to demonstrate any short or long term benefit of vitamin E supplementation on lowering oxidative stress and enhancing performance.

2.16.3 Antioxidant Vitamin Mixtures

The provitamin beta-carotene is known for its antioxidant capabilities. As an antioxidant, β-carotene was used in a few studies to investigate if it could ameliorate exercise-induced lipid peroxidation and skeletal muscle damage. Unfortunately, because β-carotene was supplied in a mixture containing other antioxidants, vitamin C and E.
it is difficult to ascertain if any positive consequence can be ascribed to beta-carotene or the other compounds or both.

This next section will therefore not explore the potential benefits of beta-carotene on exercise performance as this is not available. Instead, this section will deal with the use of antioxidant mixtures which include beta-carotene and their effect on exercise.

Kanter et al examined the effects of taking an antioxidant vitamin mixture consisting of vitamins C 1000 mg/d, vitamin E 592 mg/d, and beta-carotene 30 mg/d, for six weeks on breath pentane and serum MDA levels before and after exercise [9]. The subjects were twenty young nonsmoking males (20-29 years old) with the following makeup: five well trained, five moderately trained, and ten untrained (training state established by maximum oxygen consumption). The subjects were randomly assigned to either an antioxidant vitamin group or a placebo group. Exercise consisted of 30 minutes of running on a treadmill at 60% VO2max followed by five minutes of running at ~90% VO2max. Blood and breath samples were collected immediately after the two exercise bouts. The antioxidant supplement did not prevent the exercise-induced increase in lipid peroxidation, as reflected by the rate of pentane production or the increase in serum MDA concentration. However, ingestion of the antioxidant vitamins did result in significantly lower resting and postexercise levels of expired pentane and serum MDA. The conclusion reached by this data was that the ingestion of β-carotene, vitamin C, and vitamin E served to lower biomarkers of lipid peroxidation at rest and after exercise but did not prevent the exercise-induced increase in oxidative stress.
While this was a well done study, it still contains a few potential flaws. Although fasting blood levels were obtained for all three vitamins, subjects were excluded from the study only if their values for vitamin C or E were outside a particular range. Apparently no such range was used concerning the beta-carotene, the reasons of which were not disclosed. Moreover, daily dietary intake before vitamin supplementation was recorded, but again, no data was expressed concerning beta-carotene. Furthermore, there was no discussion of the method used to assure compliance or compliance monitoring during the course of the study. Another potential flaw of this study design was that the subjects were randomly assigned to the treatment groups and not stratified according to fitness levels. The subjects with the higher fitness levels may be subject to less oxidative stress during exhaustive exercise due to the training effect of the antioxidant enzymes.

The results of this study did provide the suggestion that supplementation with three antioxidant vitamins decreased the absolute levels of lipid peroxide biomarkers produced as a consequence of exercise. It is not clear from this data, however, whether this apparent benefit is attributable to an individual vitamin or is the cumulative effect of the vitamin mixture or both. Furthermore, the use of the particular biomarkers used in this study are not universally accepted as definitive markers of lipid peroxidation in biological systems. However, the finding that both the pentane and MDA followed similar patterns of change lends credence to their interpretation that the vitamin mixture resulted in decreased lipid
peroxidation and that exercise increased lipid peroxidation. Finally, the exercise protocol used does not allow for comparison with race performance.

Regardless of the potential faults in the above study, their results tend to favor the use of antioxidant vitamins. Conversely, another study performed by the same investigators could not duplicate the above results and is described in the next paragraph.

A prior study performed by Kanter et al undertook to evaluate similar parameters and resulted in contradictory findings as compared to the above data [139]. In this investigation, the effects of four weeks of antioxidant vitamin supplementation on serum markers of skeletal muscle damage (CK and myoglobin) and lipid peroxidation (MDA and conjugated diene formation) were evaluated. Their protocol called for the ingestion of vitamin E 1000 IU, vitamin C 1250 mg, and beta-carotene 37.5 mg on a daily basis for the four week study period. The twenty-five subjects had a mean age of 32 years and performed 30 minutes of downhill running at 65% maximum heart rate to induce eccentric exercise related damage. Samples of blood were obtained for the measurement of CK, myoglobin, MDA and conjugated dienes immediately following the exercise bout and one week later. Plasma myoglobin rose significantly in both the vitamin supplemented and placebo groups immediately following the exercise but returned to normal within 48 hours. CK levels rose by 21% and 25% in the vitamin supplemented and placebo groups respectively. These increases in CK levels in either group were not significantly different from baseline values immediately after the exercise. However, not unexpectedly, the CK levels in both the supplemented group and the
placebo group were significantly greater than their respective baseline values at 24 hours. In spite of their increases, the CK level rise at 24 hours between the two groups (supplemented vs placebo) were not significantly different. Immediately following the exercise the plasma MDA levels showed a two-fold increase in both groups, but returned to baseline within 24 hours in both groups. Conjugated diene levels decreased insignificantly in both groups and remained depressed until one week postexercise. Both the MDA and diene levels failed to achieve any statistical difference. These results indicate that four weeks of antioxidant supplementation did not afford protection from exercise-induced changes in any of the measured parameters or biomarkers. This is contradictory to the previously discussed study by the same authors. In this design however, it is very possible that the mode of exercise employed did not provide a great enough metabolic stress to promote a significant effect of vitamin supplementation (subjects exercised at ~50% \( \text{VO}_{\text{max}} \)).

One other study has been cited in the literature which explored the effects of a antioxidant mixture on exercise [10]. Eleven healthy moderately trained male volunteers, aged 18-36 years, performed a graded exercise test on a cycle ergometer for 90 minutes at 65% \( \text{VO}_{\text{max}} \) on three consecutive days. Blood samples were taken at the first and third tests, 10 minutes before the bout and at 15, 30, 60, and 90 minutes of the exercise, and at 15 minutes postexercise. The first part of the study did not include supplementation. After the final test, the subjects were placed on daily supplements consisting of vitamin E 533 mg, vitamin C 1000 mg, and \( \beta \)-carotene 10 mg for one month. At the 96
completion of one month of supplementation, the subjects repeated
the exercise and sampling procedures. In addition, after the first phase
and following the completion of the second phase, total urine output
was collected for 24 hours. This urinary collection was used to assess
oxidative damage by determining the concentration of
8-hydroxyguanosine, a marker of RNA oxidation.

They found that the plasma concentrations of all antioxidants
increased with supplementation. The urinary output of the RNA
biomarker did not change from baseline values during the three days of
exercise nor was it affected by the supplementation.

There are several problems with this data collection. The exercise
was rather mild and it is possible that more intense exercise is
necessary to damage nucleic acids. Furthermore, the use of this type of
biomarker for lipid peroxidation has been very infrequently used in
studies involving exercise. Virtually all of the research addressing the
issue of antioxidants and exercise have chosen other methods of
determining oxidative damage. The use of this particular method in
this study is questionable.

Another problem with the data collection stems from the fact
that the chosen subjects were moderately trained and training has
been known to reduce oxidative damage due to exercise, which may
hold true for RNA damage as well.

In conclusion, the equivocal results of the above described
studies stem from differences in methodology. The researchers used
different tests, different dosages of antioxidant vitamins, as well as
different durations and intensities of exercise. Nevertheless, there
seems to be promise in the antioxidant capacity of beta-carotene. This
possibility should be assessed as single supplements with a whole battery of oxidative damage assays.

2.16.4 Antioxidant Compounds

There is a conspicuous lack of any data involving virtually any type of experimentation investigating the effects of substances (as a single entity) other than antioxidant vitamins.

A review of the literature did not bring to light any human studies evaluating compounds such as glutathione (GSH). The only data found consisted of a very few studies which were performed with various antioxidant vitamin(s), or other supplements such as carbohydrates.

The only study that will be noted is one of a number of animal studies using a rat model [140]. This was a very complicated design using Han-Winstar rats that investigated the use of glutathione to decrease exercise-induced oxidative stress. In designing their experiment, the authors did note that “although antioxidant protection dependent on vitamins C and E can be enhanced by the simple use of oral supplements, increasing the reducing power of the thiol pool (primarily contributed by -SH of GSH) is far more challenging”. One of the inherent problems in performing such a study is that GSH per se is not efficiently transported into most animal cells (as noted by the authors). The researchers did cite two recent studies which provided evidence that exogenous GSH increased the endurance to physical exercise in mice which they used as a basis to devise their protocol. They chose to exogenously administer GSH intraperitoneally to the rats and exercise them to exhaustion. Sen et al also chose to
administer buthionine sulfoximine (BSO) which is known to result in glutathione deficiency.

The results of their investigation revealed that GSH administered 30 min before commencement of uphill treadmill running to exhaustion had no significant effect on endurance. Nor was there any detectable effect on the level of TBARS, used as a lipid peroxidation biomarker, at rest or after exercise. Interestingly, the artificially-induced GSH deficient rats (due to administration of BSO) had endurance to exhaustion reduced by one-half. On the basis of this finding, these investigators suggested a critical role of endogenous GSH in the circumvention of exercise-induced oxidative stress. This may have merit for further research into its role in exercise performance such that if endogenous GSH levels are depressed, ability may be impaired. They suggested that effective approaches in increasing tissue GSH pools are likely to be important in controlling exercise-induced oxidative stress and perhaps also in enhancing physical performance.

Certainly this study has some merit and was well done with adequate controls. However, as yet, it is still unknown how this data relates to human performance as the artificial conditions created in these rats is not necessarily applicable to human subjects. The implications suggested here may lead others to begin further investigations into GSH supplementation and exercise performance.

2.17 Supplementation versus Non-Supplementation

Despite the interest and activity among scientists on the relationship of exercise, free radical generation, lipid peroxidation, and antioxidant supplementation, it is still impossible to categorically
state whether there is any benefit derived from ingestion of an antioxidant supplement by the active individual. Although a large number of studies that have been performed, there are still many unanswered questions. Unfortunately, there is a conspicuous lack of uniformity in study design making study comparisons difficult at best, impossible at worst. Due to the differences in vitamin dosing and regimens, exercise protocols, supplements used, methodology and varying controls, our ability to establish a consistent opinion is doomed to failure.

One of the difficulties in interpreting the available data is lack of consistent control groups. There are few studies in the literature that actually use a valid control group.

Nevertheless, there is a trend in the available literature that suggests the benefit of antioxidant supplementation. Certainly this should be enough to continue the search for answers to this question.
CHAPTER 3
MATERIALS AND METHODS

3.1 Subjects

Twelve (12) healthy, physically fit, runners without any underlying disease were solicited to participate in the proposed study. These runners were recruited from a local running club and ranged in age from mid-20's to early 60's. Both males and females were invited to participate voluntarily. These runners are all dedicated to their sport, consistently running at least forty (40) miles per week for the past several years, and are all experienced marathoners. None of them were taking any chronic medication. Nor were they taking any vitamin or antioxidant supplements within the last thirty (30) day period prior to entering the study. They were asked to maintain a training log and continue to run at least 40 miles per week throughout the study period. Each subject was required to complete a 24 hour dietary diary just prior to each phase of the study. All of the subjects were familiar with running on a treadmill having run on them on numerous occasions prior to entering into this study.

Prior to the initiation of this research, approval for the use of human subjects was obtained from the LSU Institutional Review Board. In addition, all of the subjects signed an informed consent form prior to entering the study. Approval was also obtained from the University of Southwestern's Institutional Review Board. This was necessary as much of the testing was performed on their campus.
3.1.1 Age and Gender

At the outset of the study, the subjects consisted of twelve (12) runners: eleven (11) males and one (1) female. The ages ranged from 29 to 62 years old (average age: $45.3 \pm 8.3$ years). The lone female was 36 years old. Table 3 reveals the actual ages of each subject. Subject 05 (a male) was dropped from the study due to an overuse injury.

Table 3. Subject number and ages

<table>
<thead>
<tr>
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<th>Comments</th>
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<tbody>
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<tr>
<td>02</td>
<td>36</td>
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</tr>
<tr>
<td>03</td>
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</tr>
<tr>
<td>04</td>
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<td></td>
</tr>
<tr>
<td>05</td>
<td>44</td>
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</tr>
<tr>
<td>06</td>
<td>52</td>
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<td></td>
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<tr>
<td>12</td>
<td>45</td>
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</tr>
</tbody>
</table>

3.1.2 Methodology

Phase One: TBARS/MDA levels were utilized as the objective parameter of oxidative stress. Maximal oxygen consumption was determined via a graded exercise test (GXT). Exercise performance was measured by time to completion of a 5000 meter (5K) race. This phase
was the baseline assessment with all runners on their normal dietary intake without supplements of any kind.

**Test Day 1: Exercise performance determination**

Five kilometer race at an "all-out" effort. This race was one that was run on a local certified 5K distance. Pacing was determined by each subject monitoring their split times at mile intervals. Encouragement and incentives were provided to insure maximum effort.

**Test Day 2: Determination of maximum oxygen consumption**

This was performed within 7-10 days of the 5K race. Maximum oxygen consumption (VO\textsubscript{2max}) was performed on a Quinton 5000 treadmill using a modified Astrand protocol with the grade increasing 2% every 2 minutes while maintaining a constant speed of seven (7) miles per hour. Before initiating any testing, the Q Plex was allowed to warm-up for at least thirty minutes. Once this time had elapsed, the Q Plex was calibrated using a standardized protocol for calibration for carbon dioxide, oxygen, and volume. Barometric pressure, relative humidity, and room temperature were all included in the calibrations. Once all values were within the assigned parameters, assuring accurate determinations, the subject was allowed to begin the testing procedure. The first stage was run at only three (3) miles per hour. However, for all subsequent stages, the speed was seven miles per hour and continued until completion of the measurements. The subjects were allowed a "warm-up" period while on the treadmill prior to testing. The actual measurements were not begun until the subject indicated willingness to proceed. All of the subjects had previous experience running on a treadmill such that gaining familiarity with
the apparatus was not necessary. Each subject was required to run until exhaustion/maximal effort was attained, i.e., the point at which the subject could no longer continue in spite of encouragement. Measurement of VO\(_2\) was performed by the Quinton every 20 seconds during the entire duration of the time on the treadmill (following the “warm-up” period). The measurements were continued for at least one minute into the “warm-down” period.

Certain defined criteria were used to identify that maximum oxygen consumption was being determined. The measurement of VO\(_2\)\(_\text{max}\) satisfied the following objective criteria:

1) The running exercise used at least 50% of the total muscle mass. The exercise was continuous and rhythmical, and was done for a prolonged period of time.

2) The results were independent of motivation or skill.

3) At the highest level of exercise capacity, oxygen consumption leveled off but exercise continued at a higher intensity.

4) The measurement was performed under standard experimental conditions, avoiding stressful environments that would expose the subject to excessive heat, humidity, air pollution, or altitude.

In addition, several objective physiological parameters were included in the determination that VO\(_2\)\(_\text{max}\) had been attained. The two physiological measures that were used in this study were a respiratory exchange ratio greater than 1.0 (R = \(V_{CO2}/V_{O2}\)) and progressively diminishing differences between successive oxygen consumption measurements.
To insure that each protocol was exactly the same from one subject to the next, as well as each testing session to the next, the data and protocol was programmed into the computer. This allowed for computerized control of the necessary manipulations of the treadmill and no manual changes or control during any of the testing sessions. Additionally, each subject was weighed and measured prior to each session to insure that accurate data would be calculated during the determinations of VO$_{\text{max}}$.

**Test Day 3: TBARS/MDA analysis pre and post-exercise**

A distance of 5K (3.1 miles) was run at 85-90% race pace as determined by an average of the last 5 races at this distance (This blood draw was performed within one week of VO$_{\text{max}}$ determination.) This level of effort was chosen in order to eliminate any effects that fatigue may have on results yet insure enough exertion to result in oxidative stress. The run was performed without having engaged in any running or other aerobic activity within the previous twenty-four (24) hours. Samples of blood for TBARS/MDA analysis (under aseptic conditions) were obtained via venipuncture per Dr. LeBlanc after the subjects had been sitting quietly for at least 10 minutes prior to the 5K run. Standard tubes were used which contained EDTA as an anticoagulant. (A seated position was chosen as several of the subjects had an aversion to venipuncture in the standing position and were concerned with potential syncope.) Immediately following the completion of the 5K distance, blood samples were again obtained for TBARS analysis in a seated position. The 5K distance was run on a 400 meter track. While the run was being performed on the track, an
observer (Dr. LeBlanc) as well as the runner monitored the time by the use of a runner's watch such that correct pacing was assured. Time intervals (splits) were monitored for each lap on the track (400 meters) and made known to the subject. Splits were calculated prior to the run such that each subject was aware of the necessary time required to run each 400 meters. If deemed necessary by the observer, a practice run was done to enable the runner to become accustomed to the appropriate pace.

Rating of perceived exertion (RPE) was obtained at 5 minute intervals during the run and upon completion of the 5K run.

Immediately following the blood draw, the sample was placed into an ice chest and surrounded by ice. Upon completion of the run, the second sample was placed in the ice as well. Within 10-15 minutes of obtaining the last sample, the blood was centrifuged at 3300 revolutions per minute for twelve (12) minutes. The plasma was then removed from the sample and placed into appropriate test tube containers and placed into a freezer at minus 30 degrees Centigrade. The samples remained here until ready for TBARS/MDA analysis (within 2-3 weeks).

TBARS/MDA was determined by utilization of a lipid peroxidation assay kit using colorimetric assay. This kit was obtained from Calbiochem-Novabiochem Corporation in San Diego, California. This assay actually measured malondialdehyde (MDA). The classical determination of MDA by the thiobarbituric acid method is affected by many interfering agents. This particular assay was used because it is specific for MDA and was felt to allow greater sensitivity and reproducibility. The samples were appropriately mixed with the
reagents as instructed with the assay kit. A spectrophotometer was then used to measure the absorbance at the 586-nm wavelength. Calculation of concentration within each sample was then calculated using the equation provided by the manufacturer to determine the quantity of MDA. Every phase of the MDA determinations were done in triplicate.

**Phase Two**: In a blinded fashion, the subjects were randomly divided into two groups. This supplementation/placebo phase was performed in a double-blinded manner. One group received 25,000 IU per day beta-carotene (15 mg), while the other group received an identically appearing placebo for one month. It has previously been demonstrated that complete serum accumulation of beta-carotene takes 9-10 days [141]. Therefore, a one month phase of ingestion would insure adequate and complete serum accumulation. The subjects were instructed to avoid any additional antioxidant supplementation, most notably vitamins E, C, and beta-carotene. At the end of this one month phase, 5K race performance, VO\(_{2}\)\(_{\text{max}}\), beta-carotene and MDA production were determined as before. Compliance for ingestion of the beta-carotene/placebo was determined by counting the remaining number of pills, if any. Each subject was given a total of 42 pills in the medication bottle in both phase two and phase three. At the end of this phase, as well as phase three, compliance was 100% as documented by this counting method.

Dr. Nelson (my major professor) was in charge of randomization of the sample bottles and their contents. A registered pharmacist, Fred Mills, Jr., executive director of the La. State Board of Pharmacy, was
in charge of formulating the pills to be used during the study (both placebo and beta-carotene). Prior to completion of the data collection, only Dr. Nelson had knowledge of the actual contents of each bottle.

The beta-carotene used in this investigation was manufactured by Hoffman-LaRoche Pharmaceuticals based in Nutley, New Jersey.

**Phase Three:** This phase of the study represented the cross-over phase. The subjects remained randomly divided into the two previously assigned groups. The subjects all underwent a two-month “washout” phase to insure that beta-carotene levels had returned to normal. The two groups then ingested the other type of tablet (beta-carotene or placebo) which each subject had not had in the previous phase. One group ingested 25,000 IU (15 mg) beta-carotene daily, while the other group ingested an identically appearing placebo as before. This remained a double-blinded ingestion. At the end of a thirty day period, 5K race performance, \( \text{VO}_{\text{max}} \), and MDA production was determined as before. Once this phase was concluded, data collection was concluded.

Once the data from the three phases had been obtained, they were tabulated and compared to assess the effects of beta-carotene supplementation on: (1) exercise performance, (2) maximal oxygen consumption, and (3) oxidative stress. Additionally, assessment included determination if endurance exercise resulted in the formation of free radicals and oxidative stress in well-trained runners.

A repeated measures ANOVA was utilized to determine statistical significance for MDA and RPE results. Statistical analysis of 5K race times and \( \text{VO}_{\text{max}} \) utilized a 2-tailed paired t-test for comparison. All analyses were performed with an alpha of \( p < .05 \).

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CHAPTER 4
RESULTS

One male individual (subject 5) was dismissed from the subject population due to a running injury sustained during the course of the study. The data from this athlete were not used in any calculation of results. This reduced the original subject size to eleven (11) subjects who completed the entire protocol.

Upon completion of the data collection, the "key" listing the actual contents of bottles A and B were made known to this researcher (bottle A was first ingested, bottle B was second ingested) as listed in Table 4. This key indicates which data belong to each condition.

Table 4. Contents of Test Bottles Listed with Respective Subjects

<table>
<thead>
<tr>
<th>Bottle Number</th>
<th>Contents</th>
<th>Bottle Number</th>
<th>Contents</th>
</tr>
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<tbody>
<tr>
<td>1A</td>
<td>β-Carotene</td>
<td>1B</td>
<td>Placebo</td>
</tr>
<tr>
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<td>Placebo</td>
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<td>β-Carotene</td>
<td>12B</td>
<td>Placebo</td>
</tr>
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</table>
As previously described, there are three conditions within the design of this study. These are baseline, placebo, and β-carotene conditions. This will be indicated as such throughout this dissertation.

This study examined both baseline and placebo measures throughout each phase of the investigation. This was done to be reasonably assured that there were no carry-over effect of β-carotene from one phase to the next. In this manner, it could be validated that the beta-carotene was "washed-out" when comparing the baseline and placebo values. Statistical analysis of the data collected for the baseline and placebo phases indicated that there was no significant difference between the baseline and placebo values throughout the data collection. This was further validated by analysis of order effects (i.e., pre vs Bottle A vs Bottle B) for all factors which found that no significant order effects were discovered. Consequently, the analysis of results compared placebo vs β-carotene.

**Food diary results.** All of the subjects were required to maintain a 24-hour food diary prior to each phase of the study. This was done to provide an estimation of the daily dietary intake of beta-carotene measured in international units (IU). Moreover, this would provide assurance that there was no significant dietary changes in beta-carotene ingestion during each phase of the data collection. During the placebo phase, the mean intake of beta-carotene approximated $1175 \pm 676$ IU/day, while the mean during the beta-carotene phase was $903 \pm 1006$ IU/day excluding the 25000 IU/day supplement. It is readily apparent that there was a wide range of intake during the different phases of the study (from diet alone).
The ranges during the placebo phase and the β-carotene phases were 77-1844 IU/day and 71-3121 IU/day, respectively. The range for the beta-carotene phase does not include the supplement. The results of these diaries are listed in Tables 5 and 6. Table 6 also includes the total dietary intake of β-carotene, including the 25000 IU/day supplement, during the β-carotene phase of the study.

Table 5. Ranges of Beta-Carotene Intake (IU/day) - Diet only

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Placebo</th>
<th>β-carotene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>696 ± 602</td>
<td>1175 ± 676</td>
<td>903 ± 1006</td>
</tr>
<tr>
<td>Ranges</td>
<td>108-1941</td>
<td>77-1844</td>
<td>71-3121</td>
</tr>
</tbody>
</table>

Table 6. Beta-Carotene Daily Intake (IU/day) *

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Baseline</th>
<th>Placebo</th>
<th>β-carotene</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>452</td>
<td>1826</td>
<td>307 (25307)</td>
</tr>
<tr>
<td>2</td>
<td>293</td>
<td>1877</td>
<td>1082 (26082)</td>
</tr>
<tr>
<td>3</td>
<td>522</td>
<td>1501</td>
<td>587 (25587)</td>
</tr>
<tr>
<td>4</td>
<td>330</td>
<td>329</td>
<td>121 (25121)</td>
</tr>
<tr>
<td>6</td>
<td>462</td>
<td>664</td>
<td>444 (25444)</td>
</tr>
<tr>
<td>7</td>
<td>956</td>
<td>1844</td>
<td>3121 (28121)</td>
</tr>
<tr>
<td>8</td>
<td>1707</td>
<td>1633</td>
<td>2531 (27531)</td>
</tr>
<tr>
<td>9</td>
<td>250</td>
<td>499</td>
<td>209 (25209)</td>
</tr>
<tr>
<td>10</td>
<td>1941</td>
<td>1026</td>
<td>71 (25071)</td>
</tr>
<tr>
<td>11</td>
<td>645</td>
<td>77</td>
<td>772 (25772)</td>
</tr>
<tr>
<td>12</td>
<td>108</td>
<td>1655</td>
<td>693 (25693)</td>
</tr>
</tbody>
</table>

* The number in parentheses in the beta-carotene phase represents total daily intake (i.e., diet plus 25000 IU/day supplement).
Statistical analysis of the daily dietary beta-carotene intake (excluding beta-carotene supplementation) verified that the diet was consistent during each phase of the study. In essence, the \( \beta \)-carotene within the diet did not change and therefore did not have any impact on the results obtained in the various phases of the data collection.

**Malondialdehyde (MDA) concentration measures.** Table 7 lists the results of the MDA measurement for each subject after ingestion of placebo and following ingestion of beta-carotene. Each of these measurements were obtained from blood samples drawn before and after running a 5K distance at 85-90% effort. As indicated in the table below, pre-exercise values are listed first, while post-exercise values are listed second.

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Baseline</th>
<th>Placebo</th>
<th>( \beta )-Carotene</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.98/9.93</td>
<td>4.14/4.86</td>
<td>1.61/1.89</td>
</tr>
<tr>
<td>2</td>
<td>3.70/5.90</td>
<td>1.61/6.03</td>
<td>4.45/4.05</td>
</tr>
<tr>
<td>3</td>
<td>9.60/7.54</td>
<td>2.92/3.34</td>
<td>2.69/2.00</td>
</tr>
<tr>
<td>4</td>
<td>4.05/4.48</td>
<td>1.45/3.41</td>
<td>2.55/2.05</td>
</tr>
<tr>
<td>6</td>
<td>3.10/2.43</td>
<td>2.59/1.80</td>
<td>2.19/2.54</td>
</tr>
<tr>
<td>7</td>
<td>2.75/2.92</td>
<td>2.64/0.52</td>
<td>1.87/2.54</td>
</tr>
<tr>
<td>8</td>
<td>1.56/3.87</td>
<td>3.88/4.19</td>
<td>1.19/2.38</td>
</tr>
<tr>
<td>9</td>
<td>4.84/5.46</td>
<td>3.65/5.98</td>
<td>1.89/5.12</td>
</tr>
<tr>
<td>10</td>
<td>8.33/6.39</td>
<td>2.64/5.53</td>
<td>5.50/3.31</td>
</tr>
<tr>
<td>11</td>
<td>4.40/5.41</td>
<td>2.92/1.09</td>
<td>5.35/5.51</td>
</tr>
<tr>
<td>12</td>
<td>4.71/4.87</td>
<td>6.46/6.18</td>
<td>6.89/10.31</td>
</tr>
</tbody>
</table>
Once the above values were obtained, statistical analyses, including a repeated measures analysis of variance (ANOVA) were performed. Table 8 lists the descriptive statistics for the MDA pre-run and post-run scores.

The repeated measures ANOVA for MDA was performed and is listed in Table 9. There was no significance difference when comparing the pre to post run values for MDA collapsed across experimental conditions. In addition, the repeated measures within the treatment group failed to achieve significance. This indicates there is no significant difference between placebo and β-carotene for the MDA measures. In effect, this finding suggests that the use of beta-carotene did not alter the production of MDA, hence, free radical formation.

Table 8. Malondialdehyde descriptive statistics

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA</td>
<td>Baseline</td>
<td>4.82</td>
<td>2.38</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>3.17</td>
<td>1.38</td>
</tr>
<tr>
<td></td>
<td>Beta-Carotene</td>
<td>3.25</td>
<td>1.90</td>
</tr>
<tr>
<td>MDA</td>
<td>Baseline</td>
<td>5.38</td>
<td>2.12</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>3.90</td>
<td>2.05</td>
</tr>
<tr>
<td></td>
<td>Beta-Carotene</td>
<td>3.59</td>
<td>2.58</td>
</tr>
</tbody>
</table>

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### Table 9. Repeated Measures ANOVA for MDA results

(SS = Sum of Squares, df = degrees of freedom, sig = significance)

<table>
<thead>
<tr>
<th></th>
<th>SS</th>
<th>F</th>
<th>df</th>
<th>sig</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>.00006</td>
<td>.00002</td>
<td>1</td>
<td>0.99</td>
</tr>
<tr>
<td>Within error</td>
<td>32.61</td>
<td></td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Pre vs post</td>
<td>4.17</td>
<td>3.25</td>
<td>1</td>
<td>0.10</td>
</tr>
<tr>
<td>Within error</td>
<td>12.82</td>
<td></td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

**Five thousand meter (5K) race time measures.** Table 10 provides a listing of the 5K race times that were achieved by the subjects. The times are listed as minutes:seconds.

In order to facilitate the analysis of the numbers, the seconds in the above times were converted to hundredths of a minute. This was used in the calculation of the results. Subsequently, a 2-tailed t-test was performed for the 5K race times. Table 11 lists the descriptive statistics, while Table 12 lists the t-test results.

As may be noted from the tables, statistically significant results were obtained from the analysis of the 5K race times. The mean values for placebo and β-carotene were 19.91 ± 2.47 and 19.36 ± 2.30 respectively, in minutes with seconds converted to hundredths. The two-tailed test revealed a P value of 0.0038. This result is indicative of a significant improvement of 5K race times with beta-carotene supplementation, i.e., the runners ran faster times during the beta-carotene phase.
Table 10. Five Thousand Meter Race Results (min:sec)

<table>
<thead>
<tr>
<th>Subject No</th>
<th>Baseline</th>
<th>Placebo</th>
<th>β-carotene</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21:18</td>
<td>20:55</td>
<td>21:02</td>
</tr>
<tr>
<td>2</td>
<td>20:14</td>
<td>20:30</td>
<td>20:02</td>
</tr>
<tr>
<td>3</td>
<td>17:22</td>
<td>17:48</td>
<td>17:09</td>
</tr>
<tr>
<td>4</td>
<td>17:15</td>
<td>17:20</td>
<td>17:17</td>
</tr>
<tr>
<td>6</td>
<td>19:05</td>
<td>18:43</td>
<td>18:21</td>
</tr>
<tr>
<td>7</td>
<td>18:46</td>
<td>18:49</td>
<td>18:32</td>
</tr>
<tr>
<td>8</td>
<td>16:30</td>
<td>16:59</td>
<td>16:24</td>
</tr>
<tr>
<td>9</td>
<td>25:20</td>
<td>25:14</td>
<td>24:24</td>
</tr>
<tr>
<td>10</td>
<td>18:27</td>
<td>19:40</td>
<td>18:30</td>
</tr>
<tr>
<td>11</td>
<td>22:03</td>
<td>22:51</td>
<td>21:17</td>
</tr>
<tr>
<td>12</td>
<td>18:30</td>
<td>20:12</td>
<td>19:58</td>
</tr>
</tbody>
</table>

Table 11. 5K Descriptive Statistics (seconds converted to 100ths)

<table>
<thead>
<tr>
<th>N</th>
<th>Mean</th>
<th>Std. Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>11</td>
<td>19.53</td>
</tr>
<tr>
<td>Placebo</td>
<td>11</td>
<td>19.91</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>11</td>
<td>19.36</td>
</tr>
</tbody>
</table>
Table 12. 5K 2-tailed t-test results

(Diff. = difference, df = degrees of freedom, prob. = probability)

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>Mean Diff.</th>
<th>Error of Diff.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>19.91</td>
<td>2.47</td>
<td>0.55</td>
<td>0.15</td>
</tr>
<tr>
<td>β-carotene</td>
<td>19.36</td>
<td>2.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>t-value</strong></td>
<td></td>
<td></td>
<td>3.78</td>
<td></td>
</tr>
<tr>
<td><strong>df</strong></td>
<td></td>
<td></td>
<td>10</td>
<td></td>
</tr>
<tr>
<td><strong>2-tail prob.</strong></td>
<td></td>
<td></td>
<td>0.0038</td>
<td></td>
</tr>
</tbody>
</table>

Maximum oxygen consumption (VO_{max}) measures. Table 13 supplies the data for the results of the VO_{max} testing for the placebo and beta-carotene phases of the study. The VO_{max} was measured in ml/kg/min. Table 14 provides the descriptive statistics for the measurement of VO_{max} for each subject. The mean values for the placebo and β-carotene phases were 59.96 and 61.46 respectively. These statistics are followed by Table 15 which presents the results of a 2-tailed t-test analysis. The results of the 2-tailed t-test failed to produce any statistically significant improvement of the VO_{max}. Consequently, it may be surmised that the supplementation of beta-carotene had no effect on maximum oxygen consumption of any of the subjects as compared to placebo. The VO_{max} remained unaffected by the use of beta-carotene.
### Table 13. Maximum Oxygen Consumption Results (ml/kg/min)

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Baseline</th>
<th>Placebo</th>
<th>β-carotene</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>59.8</td>
<td>63.5</td>
<td>59.9</td>
</tr>
<tr>
<td>2</td>
<td>49.4</td>
<td>56.8</td>
<td>57.5</td>
</tr>
<tr>
<td>3</td>
<td>63.9</td>
<td>63.2</td>
<td>66.2</td>
</tr>
<tr>
<td>4</td>
<td>67.9</td>
<td>67.7</td>
<td>69.6</td>
</tr>
<tr>
<td>5</td>
<td>57.8</td>
<td>54.8</td>
<td>66.8</td>
</tr>
<tr>
<td>6</td>
<td>75.5</td>
<td>79.4</td>
<td>71.2</td>
</tr>
<tr>
<td>7</td>
<td>66.2</td>
<td>68.3</td>
<td>69.2</td>
</tr>
<tr>
<td>8</td>
<td>46.5</td>
<td>38.9</td>
<td>47.2</td>
</tr>
<tr>
<td>9</td>
<td>55.3</td>
<td>64.0</td>
<td>57.8</td>
</tr>
<tr>
<td>10</td>
<td>52.7</td>
<td>54.0</td>
<td>53.2</td>
</tr>
<tr>
<td>11</td>
<td>60.3</td>
<td>49.0</td>
<td>57.5</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 14. Maximum Oxygen Consumption Descriptive Statistics

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>Std. Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>11</td>
<td>59.57</td>
<td>8.54</td>
<td>2.57</td>
</tr>
<tr>
<td>Placebo</td>
<td>11</td>
<td>59.96</td>
<td>10.87</td>
<td>3.28</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>11</td>
<td>61.46</td>
<td>7.69</td>
<td>2.32</td>
</tr>
</tbody>
</table>
Table 15. 2-tailed t-test for VO2 max

(Diff. = difference, df = degrees of freedom, prob. = probability)

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>Mean Diff.</th>
<th>Error of Diff.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>59.96</td>
<td>10.87</td>
<td>-1.5</td>
<td>1.89</td>
</tr>
<tr>
<td>β-carotene</td>
<td>61.46</td>
<td>7.69</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

t-value  -0.79
df  10
2-tail prob.  0.45

Rating of Perceived Exertion (RPE) measures. Table 16 lists the data collected on the RPE of the subjects during the 5K run at 85-90% effort. Each RPE was taken every five (5) minutes during the run with the final RPE taken immediately upon completion of the run. Table 17 lists the descriptive statistics for each RPE at the various five minute time intervals. All of the subjects obtained an RPE value for 20 minutes and/or the end of the run. However, not all of them obtained a 25 minute RPE. For ease of calculation, the analyses were limited to the intervals at 5, 10, 15, and 20 minute RPEs.

It may be gleaned from Table 18 that there was no significant difference between the placebo and beta-carotene phases of the study. The comparison of these two phases failed to demonstrate any beneficial effect of the use of beta-carotene on RPE values. Not surprisingly, a statistically significant difference was found between the RPE values comparing the values obtained in the pre and post phases. Therefore, the use of beta-carotene failed to be of benefit when considering RPE values in this study.
### Table 16. 5K Run RPE @ 85-90% effort (5 minute intervals/end)

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td>13/15/17/18/18</td>
<td>12/13/13/14/14</td>
<td>12/13/14/16</td>
</tr>
<tr>
<td><strong>Placebo</strong></td>
<td>9/11/14/14/15</td>
<td>11/13/13/14/14</td>
<td>14/14/15/15</td>
</tr>
<tr>
<td><strong>β-carotene</strong></td>
<td>12/14/17/17/17</td>
<td>9/10/11/12/12</td>
<td>12/13/13/14</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>4</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td>11/13/15/15</td>
<td>13/13/14/14</td>
<td>13/13/13/15</td>
</tr>
<tr>
<td><strong>Placebo</strong></td>
<td>11/14/15/15</td>
<td>13/13/14/14</td>
<td>9/11/13/13</td>
</tr>
<tr>
<td><strong>β-carotene</strong></td>
<td>10/11/12/12</td>
<td>11/11/12/13</td>
<td>9/9/11/11</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td>12/13/13/13</td>
<td>12/13/14/15/17</td>
<td>12/13/13/15/15</td>
</tr>
<tr>
<td><strong>Placebo</strong></td>
<td>14/14/16/16</td>
<td>11/13/13/13/13</td>
<td>11/11/11/13/13</td>
</tr>
<tr>
<td><strong>β-carotene</strong></td>
<td>12/13/13/14</td>
<td>12/12/12/12/12</td>
<td>12/13/13/15/15</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td>9/11/11/11/12</td>
<td>13/13/15/15/17</td>
</tr>
<tr>
<td><strong>Placebo</strong></td>
<td>8/10/11/13/13</td>
<td>13/15/17/18/18</td>
</tr>
<tr>
<td><strong>β-carotene</strong></td>
<td>7/9/11/13/14</td>
<td>12/14/16/17/18</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>Mean</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>---</td>
<td>-------</td>
</tr>
<tr>
<td><strong>RPE 5K 5 minutes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>11</td>
<td>12.00</td>
</tr>
<tr>
<td>Placebo</td>
<td>11</td>
<td>11.27</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>11</td>
<td>10.73</td>
</tr>
<tr>
<td><strong>RPE 5K 10 minutes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>11</td>
<td>13.00</td>
</tr>
<tr>
<td>Placebo</td>
<td>11</td>
<td>12.64</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>11</td>
<td>11.73</td>
</tr>
<tr>
<td><strong>RPE 5K 15 minutes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>11</td>
<td>13.73</td>
</tr>
<tr>
<td>Placebo</td>
<td>11</td>
<td>13.82</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>11</td>
<td>12.82</td>
</tr>
<tr>
<td><strong>RPE 5K 20 minutes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>11</td>
<td>14.55</td>
</tr>
<tr>
<td>Placebo</td>
<td>11</td>
<td>14.36</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>11</td>
<td>13.64</td>
</tr>
</tbody>
</table>
Table 18. Repeated Measures ANOVA for RPE results

(SS = Sum of Squares, df = degrees of freedom, sig = significance)
(F = F-test score)

<table>
<thead>
<tr>
<th></th>
<th>SS</th>
<th>F</th>
<th>df</th>
<th>sig</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>13.92</td>
<td>2.36</td>
<td>1</td>
<td>0.16</td>
</tr>
<tr>
<td>Within error</td>
<td>58.95</td>
<td></td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Pre vs post</td>
<td>114.58</td>
<td>30.12</td>
<td>3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Within error</td>
<td>38.05</td>
<td></td>
<td>30</td>
<td></td>
</tr>
</tbody>
</table>

In addition, it should be noted that upon completion of the data collection, each subject was asked the following question: "Depending on the way you felt during your running, training, racing, and recovery, which bottle (A or B) contained β-carotene?" The results were as follows: of the eleven (11) subjects who completed the study, seven (7) correctly named the proper bottle, three (3) incorrectly identified the proper bottle, while one subject stated that no differences were noted with either of the two bottles. This indicates that 64% of the subjects could detect a beneficial effect while on the beta-carotene allowing them to correctly identify this pro-vitamin. On the other hand, 27% noted a beneficial effect on the placebo, and only one subject noted no beneficial effect with either bottle. This allows a strong suggestion that there was improvement with the use of beta-carotene, albeit a subjective one.
In summary, the results of this investigation demonstrate that beta-carotene supplementation benefited only one phase of this study, i.e., 5K race times. The only statistically significant finding was in the 5K race results as compared to placebo. No significant difference was found with the use of beta-carotene in either decreasing oxidative stress (MDA) or improving maximum oxygen consumption. Likewise, there was no substantial change in RPE values with the ingestion of beta-carotene. All of these results were found with a consistent daily dietary intake of beta-carotene (excluding supplementation).
In the last two decades, there has been considerable research in the area of free radicals and their reactivity. The oxidative effects imposed on cellular and subcellular structures have been well described. Although some free-radical induced effects may be deemed beneficial to the aerobic organism, many of them have been found to be harmful with subsequent undesirable reactions.

In an effort to combat these deleterious consequences, various biological systems have evolved to protect oxygen requiring species (in particular, the human organism) from free radical damage. These antioxidant defenses in biological systems are represented by enzymatic and non-enzymatic mechanisms. At times, these defenses may prove adequate in subverting radical-induced mayhem. However, due to the increased oxygen consumption which occurs in an exercising person, these antioxidant defenses may be inadequate [96, 108, 116, 118, 119, 120, 121]. Consequently, there has been considerable scientific research in free radical and antioxidant biology.

Much of this investigation has focused on the antioxidants vitamin E, vitamin C, and beta-carotene. In fact, there has been rather intense research in studying the antioxidant effects of vitamin E and vitamin C alone, or in combination [31, 94, 127, 128, 130, 131, 134, 135, 136, 137]. However, the investigation of the antioxidant effects of beta-carotene has been scanty. In actuality, there exist only a few studies in the literature that have evaluated the effects of β-carotene
during physical activity. Unfortunately, all of the aforementioned studies were performed in combination with vitamin C and E, making the individual effect of beta-carotene impossible to determine [7, 8, 9, 10]. Therefore, in spite of various studies in this area, no research is available to ferret out the antioxidant effects of beta-carotene during exercise. This void was recognized, serving as a nidus for this investigation. Therefore, an attempt has been made to shed some light in this area of antioxidant research, i.e., the effects of beta-carotene (alone) during exercise.

Encouraged by a pilot study of three runners (described in chapter 1), which suggested a beneficial effect by the supplementation of beta-carotene, a more intense investigation was devised. The main purpose of this study was to evaluate the effects of beta-carotene on three parameters. These parameters included evaluation of exercise performance, exercise-induced oxidative stress and maximum oxygen consumption.

Accordingly, three hypotheses were tested and are as follows:

1. During the running of a 5K distance, even well-trained runners will see an increase in biomarkers of oxidative stress. This will be identified by increases in serum MDA concentrations.

2. Oxidative stress is produced during a 5K race/run. This stress may be reduced by beta-carotene. As a result of supplementation, exercise performance will be enhanced, as measured by improved 5K race times, compared to the normal daily dietary intake of β-carotene in well-trained runners.

3. Beta-carotene supplementation will improve maximum oxygen consumption as a consequence of a reduction in oxidative damage.
5.1 Hypothesis 1: Oxidative Stress

It is a well known phenomena that exercising persons must increase oxygen consumption to coincide with increased oxygen demand. Additionally, it has been previously noted that 4-5% of consumed oxygen is transformed into free radicals [35]. Therefore, it would appear that a well-trained runner would generate more free radicals during exercise compared to an inactive state. Conversely, it may be stated that due to adaptive responses, the antioxidant defense mechanisms would be more keenly developed to resist this increase in generation of free radicals. As such, this would result in little or no increase in biomarkers of oxidative stress (i.e., MDA concentrations).

The use of MDA as the biomarker of choice was an important consideration for the measurement of free radical formation. MDA concentrations have been found to be a very sensitive assay in the detection of peroxidation [106]. Moreover, this assay is frequently cited in the literature and, accordingly, would provide this current research with comparable values from previous studies [9, 94, 110, 113, 131, 134, 136, 139]. Simply stated, the determination of MDA concentration changes has become a standard in exercise literature and allows comparison of the results obtained in this study to previous work done in this field. Hence, the use of MDA in the testing of the first hypothesis.

Hypothesis 1 states that there would be an identifiable increase in oxidative stress, hence, an increase in MDA levels. In effect, this hypothesis asserts that the normal antioxidant defenses are inadequate in preventing increased oxidative stress during exercise.
However, this increase could be thwarted by the use of an antioxidant such as beta-carotene in supplemental fashion (25000 IU/day).

In this investigation, a distance of 5000 meters was chosen as representative of a long distance which could be easily run by these runners (all of whom were consistently running forty miles per week for quite some time prior to and during the data collection). This 5K distance (designated as 5K run in distinction to a 5K race) would be a sufficient physical challenge at 85-90% effort to induce oxidative stress while avoiding an exhaustive state. It was felt that this would avoid any terminal stresses associated with an all-out effort with potentially confounding attributes.

The results of the MDA blood samples during the placebo and beta-carotene phases of the study may be seen in Table 7 while the MDA descriptive statistics are shown in Table 8. In accordance with the first hypothesis, the anticipated results were: 1) an increase in MDA following the 5K run when compared to pre-run values, indicative of the presence of oxidative stress; 2) decreased MDA with the supplementation of beta-carotene in pre-run scores; and, 3) a decreased elevation of post-run MDA scores with beta-carotene supplementation.

The actual pre to post comparison of MDA results did reveal an increase in mean placebo values (3.17 to 3.90 μM), and an increase in mean beta-carotene phase values (3.25 to 3.59 μM). These values are consistent with values (range = 2.5 to 5.5 μM) found in the literature [9, 10, 139]. The oxidative stress posed by the 5K run would be reflected in the increased production of free radicals and subsequent rise in MDA concentrations regardless of the phase of the study.
Subsequently, these MDA results were analyzed using a repeated measures ANOVA and are displayed in Table 9. There is no statistically significant difference between placebo and beta-carotene for MDA measures. However, it may be noted that there is a trend \( (P = 0.10) \) toward an increase in oxidative stress when comparing the pre-run vs post-run values for both treatments.

Therefore, it may be stated that the measurement of MDA failed to demonstrate any significant difference between the placebo versus beta-carotene phases of the study. This implies that the supplementation of beta-carotene exerted no beneficial effect on oxidative stress in these runners. In essence, the results demonstrate that the beta-carotene did not significantly exert any effect on the formation of free radicals which may be generated during a 5K run.

Although these data did not reveal a significant effect with the use of beta-carotene, there are other considerations that must be entertained. For example, it is possible that MDA is a poor measure of free-radical formation, even though MDA is a well-known, frequently described, and commonly used biomarker of oxidation used to detect free radical formation. Regardless of its frequent citation in the literature, it is not without its criticisms and weaknesses as a measure of oxidative stress and the formation of free radicals \([9, 33, 105]\). For example, the measurement of MDA is a nonspecific assay. In addition, a variable amount of the MDA produced in the thiobarbituric acid test is formed during the acid-heating phase of the assay \([9, 105]\).

Nevertheless, in this present research, this was the only feasible method which could be employed. It was not possible in this
investigation to utilize any other form of free radical/oxidative stress measurement. This was the consequence of many factors, such as technical expertise, equipment availability, ease of use, cost factors and funding sources. An alternative approach would have been to determine the presence and quantity of a second biomarker of oxidative stress (e.g., pentane levels) to verify the results obtained by the MDA values. Unfortunately, this was not possible for this investigation because of the factors noted above.

Further, it is possible that the beta-carotene may have some local impact, particularly at the tissue level at the lowest oxygen pressures [4, 5]. In this investigation, measurements were confined to whole blood which does not represent tissue samples. Beta-carotene could have some local effect which may alter the production of free radicals and/or the efflux of them within the tissues. The alteration and detection of either the production of free radicals or their efflux from the tissues is an important consideration as to this vitamin’s effectiveness and its consequence. Certainly, the measurement of these two processes, i.e., production vs efflux, would be quite difficult. As such, the use of whole blood methodology is not representative of tissue samples and may be a poor reflection of beta-carotene effects. At present, there is no easy remedy to this question in the exercising athlete. There is no available non-invasive method which could be utilized to monitor tissue levels (either free radical production or efflux) during physical activities. The use of currently available techniques such as arteriovenous catheters, certainly could not be used in a blinded fashion nor could they be used without having a major influence on the physical activity.
5.2 Hypothesis 2: Exercise Performance

It has been shown in previous investigations that physical activity does produce oxidative stress [9, 47, 92, 93, 94]. This finding was marginally supported by an increase in MDA levels following a 5K run in this study. In accordance with this finding, the second hypothesis states that this stress may be reduced by the antioxidant vitamin β-carotene. As a consequence of supplementation, exercise performance would be enhanced, as measured by improved 5K race times (in distinction to 5K run times). This supplementation would aid the subjects as compared to the normal daily dietary intake of beta-carotene in these well-trained runners. It is noteworthy to state that the literature has previously established that runners often have decreased levels of beta-carotene [11]. In addition, this finding was also supported by my pilot study (as documented in chapter one). Table 10 lists the times for the 5K race performed by each of the participants in the study while Table 11 displays the 5K descriptive statistics in minutes (with seconds converted to hundredths). The race times during the placebo phase were $19.91 \pm 2.47$, while the race times during the beta-carotene phase were $19.30 \pm 2.30$ minutes. This is the expected finding anticipated by the second hypothesis. When the results were placed under the scrutiny of statistical evaluation via a 2-tailed t-test, this decrease in 5K race times was found to be significant with $P = 0.0038$ (Table 12). Consequently, these results support the second hypothesis which stated that exercise performance would be enhanced with beta-carotene supplementation.

In spite of these positive results, the mechanism by which this is accomplished is not clear. The MDA analysis previously discussed
failed to demonstrate a significant difference with the use of beta-carotene in an attempt to decrease oxidative stress and free radical formation. It was proposed by the second hypothesis that 5K race times would improve as a result of decreased oxidative stress by this vitamin. Therefore, although the race times did improve, this may not have been a consequence of a reduction in free radical-induced oxidative stress. It is possible that the beta-carotene may exert a positive influence other than its expected role as an antioxidant which has not yet been determined. Nevertheless, this data suggests that beta-carotene supplementation does exert a beneficial effect on exercise performance as demonstrated by a significant improvement in 5K race times. The exact mechanism responsible for this improvement, however, is unknown.

5.3 Hypothesis 3: Maximum Oxygen Consumption

The third hypothesis stated that supplementation with β-carotene would reduce cellular and subcellular oxidative damage. Consequently, beta-carotene supplementation would improve maximum oxygen consumption (VO$_{2\text{max}}$). This theory was supported by previous data documenting the antioxidant effects of β-carotene, particularly at the tissue level \([3, 4, 5]\).

Table 13 supplies the results of the VO$_{2\text{max}}$ testing during the placebo and beta-carotene phases of the study while Table 14 displays the descriptive statistics. When inspecting Table 14, it may be noted that VO$_{2\text{max}}$ did rise in the beta-carotene phase as compared to the placebo portions of the study. The VO$_{2\text{max}}$ during the placebo phase was 59.96 ± 10.87 ml/kg/min. As indicated, the use of beta-carotene
succeeded in raising the value to 61.46 ± 7.69 ml/kg/min. This was the finding suggested by the third hypothesis. This improvement could be brought about by a reduction in cellular and subcellular oxidative damage. A decrease in this damage would improve cellular respiration which would translate into improved oxygen consumption. However, the significance of this result was not borne out with statistical analysis. Table 15 provides the 2-tailed t-test for VO$_{2\text{max}}$. It may be seen by this analysis that no statistical significance was found with the use of beta-carotene as compared to placebo.

Several factors must be considered in reference to this data analysis however. The most prominent in this regard is the fact that the measurement of VO$_{2\text{max}}$ may be too gross a method to determine the effects of beta-carotene at the cellular level. VO$_{2\text{max}}$ is a function of cardiac output and the extraction of oxygen from the blood. Of these two, the one with the greatest capacity to change with chronic aerobic training is the cardiac output via increasing stroke volume. All of the runners in this investigation had been training for many years. Consequently, each of these athletes had reached their maximum stroke volume prior to entering this study. Furthermore, the change in oxygen extraction would be small and, with only eleven subjects, probably outside the power of the test to detect a significant difference. However, improvement of only a slight magnitude, while not statistically significant, may still dwell within the realm of a beneficial impact when comparing tenths of minutes in relation to exercise performance.
As mentioned under hypothesis one, beta-carotene may exert its antioxidant properties at the tissue level within the confines of the lowest oxygen pressures. As such, a measurement such as VO₂max would be unable to detect such a minute detail. The effects deep within tissues may provide a supportive rationale for the use of beta-carotene, however, the measurement of VO₂max will not provide sufficient information to effectively acknowledge this determination. This is not to say that the assessment of VO₂max was fruitless or without foundation. The measurement of VO₂max is the standard while analysis at a cellular level is impractical and too invasive.

5.4 Rating of Perceived Exertion (RPE) Measures

In addition to collecting the data to explore the possibilities of the three hypotheses, the rating of perceived exertion was determined for each phase of the study. This data was accumulated to determine if there might be any subjective benefit from the use of beta-carotene. It was theorized that as a consequence of its antioxidant properties, supplementation of beta-carotene would decrease perceived exertion during the 5K run. Therefore, RPE was obtained during five (5) minute intervals of the 5K run.

Table 16 provides the results of the RPE collection during the 5K run at 85-90% effort, while Table 17 provides the descriptive statistics for each of the five minute intervals. Table 18 demonstrates the repeated measures ANOVA for the RPE results. Not unexpectedly, the pre to post RPEs were significantly different within the subjects. This would seem logical. In particular, the emphasis was on the RPE of the treatment condition, i.e., the beta-carotene phase. When this variable
was calculated, there was no apparent benefit from the ingestion of beta-carotene. In other words, there was no statistically significant difference in RPEs during the treatment phase of the study with the use of beta-carotene when compared to baseline or placebo.

5.5 Food Diary Measures

Food diaries were obtained from each participant prior to each phase of the study. This was obtained to estimate the intake of beta-carotene for each subject measured in international units. This was obtained in order to determine if there was any notable difference in the intake from one subject to another and from one phase to the next. It was quite possible that some athletes may be ingesting more beta-carotene containing foods than another. This may result in a large discrepancy during the study depending on the ingested amount. This food diary would help evaluate this potential dilemma. In reviewing the results of the food diaries as listed in Tables 5 and 6, it is apparent that no trend is present. In fact, there is a very wide range of ingestion among all the subjects during each phase of the study. However, the collection of this data was useful. The diaries did verify that there was no significant difference in the daily dietary consumption of β-carotene during either the placebo or supplemental phase of the study. These data suggest that the only variable in the daily intake of beta-carotene occurred during the supplemental phase. Therefore, the notion that the dietary intake remained stable during the data collection is inferred. However, it should be made clear that there is a marked variation in the daily dietary intake of beta-carotene in these subjects. In addition, the dietary intake was much less than daily intake described in the literature. The American Cancer Society
recommends a dietary intake of at least 5.0 mg/day [142]. The amount of beta-carotene ingested by these subjects varied from a low of 0.04 mg/day to a high of only 1.92 mg/day. Previous information collected from a large scale study of 121,700 subjects revealed that 75% ingested more than 3850 IU/day (2.31 mg/day) [142]. Therefore, although there was no significant difference in dietary beta-carotene intake among these runners (in my study) as a whole, there was marked individual variation. Therefore, it may be generally stated that they were deficient in their daily intake of beta-carotene according to their dietary recall. This suggests that the runners in this data collection would benefit from beta-carotene supplementation.

5.6 Subjective Evaluation Follow-up Question

During the course of this investigation, it was noted that most of the subjects could detect some effect of the double-blinded ingestion. The vast majority of the runners felt that they could note a beneficial effect on their training and recovery by the use of one of the unnamed bottles. Therefore, as a follow-up to the ratings of perceived exertion, one last question was asked of each subject at the conclusion of the data collection. Each subject was asked to determine the content of Bottles A and B. They were asked to evaluate how they felt during each phase of the study and compare the effort of running, training, racing, and the manner in which they recovered from these events. Interestingly, the subjects overwhelmingly correctly identified the bottle with the beta-carotene (recall that this was a double-blinded study). Seven of the eleven subjects (64%) stated that they felt better while on the beta-carotene and felt that their training, running, and recovery was quicker and easier. Additionally, they felt that their race...
performances were enhanced with the beta-carotene supplementation. On the other hand, a minority of 27% (3/11) incorrectly stated that the placebo was the bottle that contained beta-carotene. Only one subject (9%) felt that he could detect no difference in either bottle during the course of the study. Therefore, it is quite apparent that in deference to a lack of actual statistically significant analysis (with exception of the improvement in 5K race performance), the majority of the subjects felt better while on the beta-carotene.

Moreover, it may be noted from the 5K race times (Table 10), that ten of the eleven subjects actually improved. Although these differences are rather small, and the other measured parameters failed to achieve statistical significance, they may be representative of practicality rather than one related to statistics. In sports where the difference between winning and losing is measured in hundredths of a second, any improvement is noteworthy and beneficial. Although data has been presented from the literature and from my pilot study that supports the notion that athletes may be relatively beta-carotene deficient, certainly this is not true for all athletes. It is quite possible that the athletes who demonstrated the most improvement represented those with the least amount of total body beta-carotene. Consequently, these would be the athletes who had the most to gain by the supplementation phase of this study.

An interesting final note is in order. Of the eleven subjects in this investigation, several of them (seven) have continued to ingest the supplemental beta-carotene. Three of these have continued the supplementation on a daily basis. The remaining four have ingested the additional 25000 IU/day on an intermittent basis. Those who have...
ingested the beta-carotene on an intermittent basis are using it during periods of increased training or increased intensity in the workouts. These four indicate that they feel better supplementing their diets and seem to recovery easier as well. Likewise, the three subjects who are using supplementation of beta-carotene on a continual daily basis state that they seem to note a beneficial effect. They also indicate that they are able to run through their workouts easier, train harder, and recover faster. Two of these latter subjects report that their race times have noticeably improved. Both of them have noted marked improvement in their ability to run faster times. One subject in particular (29 years old, running competitively since age 14) has run personal bests on two separate occasions for a 5K distance. This subject categorically states that the beta-carotene has played a major role in his improvement.

Certainly, at this point, some of these reports may be the eventuality of a psychological benefit. This is a less likely occurrence, however. To date, there are no published reports in the literature to indicate that beta-carotene has any mood-altering capabilities or other psychological effects. Additionally, these are the athletes who may respond to the use of beta-carotene while the others may not due to a possible deficient state. Moreover, it is possible that these subjects may have less well developed enzymatic antioxidant mechanisms to combat free radical stress and therefore receive the most benefit.

Regardless of the precise explanation, several subjects continue to use beta-carotene as part of their training regimen. They perceive a worthwhile effect and are willing to continue its use.
CHAPTER 6
SUMMARY, CONCLUSION, FUTURE DIRECTIONS

6.1 Summary

Free radicals and antioxidants have become a frequently discussed topic among both lay and professional journals in recent years. Evidence has been accumulating in the scientific literature that suggests that free radicals play a detrimental role in cellular and subcellular events. This has been particularly noted with aerobic respiration and, as such, is a normal phenomenon. During exercise and increased oxygen consumption, these detrimental events may be magnified due to increased free radical generation. The human body is endowed with mechanisms to combat the bombardment of free radicals, i.e., enzymatic and non-enzymatic means. Certain citations in the literature suggest that the enzymatic mechanisms may not be adequate in the exercising individual. Therefore, there may be increased reliance on non-enzymatic mechanisms to protect the cells from radical-induced destruction. Likewise, there are additional citations suggesting that the exercising person may bolster the existing antioxidant defenses with increased ingestion of antioxidants such as vitamin C, vitamin E, and beta-carotene.

A review of the literature did reveal multiple studies which investigated the effect of antioxidants on free radical-induced damage. The vast majority of these studies were performed using vitamin C and/or vitamin E. Only a handful of articles were found which undertook any investigation of beta-carotene's antioxidant properties.
However, all of the data were collected within a mixture of vitamins C and E. Understandably, this renders the isolated effects of beta-carotene impossible to determine. As a result, the antioxidant effects of beta-carotene alone served as the investigative mission for this data collection.

This study began with the following three hypotheses:

1. During the running of a 5K distance, even well-trained runners will see an increase in biomarkers of oxidative stress. This will be identified by increases in serum MDA concentrations.

2. Oxidative stress is produced during a 5K race/run. This stress may be reduced by beta-carotene. As a result of supplementation, exercise performance will be enhanced, as measured by improved 5K race times, compared to the normal daily dietary intake of β-carotene in well-trained runners.

3. Supplementation with beta-carotene will reduce cellular and subcellular oxidative damage. Consequently, beta-carotene supplementation will improve maximum oxygen consumption.

The data was collected using eleven (11) subjects who were all well trained and running at least forty (40) miles per week. In order to assure quality control, this study adopted a double-blinded cross-over design. These runners were randomly assigned to ingest either placebo or beta-carotene for at least four (4) weeks prior to data collection for the particular phase. This was followed by the cross-over condition. At the completion of each phase, measurements were obtained for MDA concentrations before and after a 5K run at 85-90% effort, race times for a 5K race, and VO$_{2\text{max}}$. An initial baseline analysis was also
performed but was not used in the final calculations as there was no statistically significant difference between the results obtained in either the baseline or placebo phases as expected. Moreover, food diaries collected in the twenty-four (24) period prior to beginning each phase failed to note any statistically significant difference in the amount of beta-carotene consumed from dietary sources (excluding supplementation). In other words, the only difference between the beta-carotene content in the diet in either phase of the study was the supplemented amount.

Once all phases were completed and the data collected, the actual contents of bottles A and B were made known to this investigator. Only then were the data analyzed for statistical significance.

Statistical analysis failed to demonstrate any verification of the first and third hypotheses. There was no statistically significant difference in the MDA concentrations with or without the use of beta-carotene (25000 IU/day) as compared to placebo (first hypothesis). In addition, determination of VO_{max}, comparing placebo vs beta-carotene supplementation, failed to reach any significant difference in the obtained values (third hypothesis). Additionally, the assessment of RPE during each phase did not suggest any difference when comparing placebo vs beta-carotene during the 5K run.

Significance was found in the improvement of 5K race performance however (second hypothesis). The data did indicate that the supplementation of beta-carotene resulted in faster race times when compared to placebo (P = 0.0038). This decrease in race times
was due to the beta-carotene supplementation alone as dietary sources remained constant throughout the study.

Lastly, subjective evaluation of the effectiveness of the beta-carotene was implied by the noticeable fact that sixty-four percent (64%) of the subjects were able to correctly identify the contents of the bottles. Furthermore, seven of the subjects (64%) opted to continue the additional beta-carotene on either a daily or intermittent basis due to their reported beneficial effects by its use.

6.2 Conclusions

The results of this investigation support hypothesis two, i.e., that 5K race performance could be enhanced by beta-carotene supplementation. In addition, a majority of the subjects noted a subjective advantage from the supplementation. However, the data failed to support either hypothesis one or three. No statistically significant increase in MDA concentration following exercise was found, nor was there any reduction of MDA by the use of beta-carotene. Furthermore, there was no significant gain with the supplementation of beta-carotene as a means of improving VO$_{\text{max}}$.

Therefore, although the data does support the use of beta-carotene supplementation as a means of improving 5K race performance, the mechanism by which this occurs has not been shown.

Although this study failed to support two of the three hypotheses, valuable information was garnered by this research. Heretofore, no data were available investigating the antioxidant effects of beta-carotene alone. The information presented here is the first to
provide data in this unexplored territory. Race performance was improved, yet the proposed reduction in oxidative stress was not found. Likewise, the improvement could not be attributed to an increase in VO$_{2\text{max}}$. Regardless of the mechanism, a majority of the subjects elected to continue the beta-carotene supplementation providing a subjective testament to its usefulness. The data collected here raises multiple issues and provides insights into various directions for future investigation. Any one of these may be pursued in the analysis of beta-carotene's ergogenic possibilities.

6.3 Future Directions

A reasonable thought may combine the belief that free radicals are damaging to our cells and that these same destructive species may be counteracted by antioxidants. Numerous studies attest to the utility of nutritional antioxidant supplementation, while on the other hand, there are contradictory results from other numerous studies as well. This does not necessarily indicate that the existing information is incorrect, rather that more data is needed. Just because a substance does not alter the VO$_{2\text{max}}$ does not mean it has no effect on sports performance.

Presently available studies differ in many aspects, including study design, type and number of subjects, methodology, and types and dosages of antioxidants. The results of these studies should be used as a springboard to further research in this area and to facilitate the accumulation of data. There is a definite trend toward the implication that free radicals present a formidable force attacking the body's defenses. Certainly, it would seem prudent to combat these free
radical species in a safe and expedient manner such as in the use of antioxidants. As the natural defenses against free radical bombardment are bolstered, the ability of the body to perform and interact with its environment is enhanced. Ostensibly, this would promote the ability to perform exercise with less damage and improved results. This type of speculation implores the continuation and extension of current research to provide answers to the question of promotion of nutritional antioxidants in fighting free radical degradation and enhancing performance.

The results of this investigation provide evidence in support of the antioxidant properties of beta-carotene. However, the data collected here do not allow supporting information to explain its mode of action or its effects. Consequently, it can only be theorized as to the exact etiology of its beneficial effect during race performance. The development of a foundation for the obtained results may serve as an impetus to continue this research. Some of these findings will allow the initiation of other thoughts and concerns in the antioxidant properties of beta-carotene and the manner in which this knowledge is gained.

For example, one of the surprising and unanticipated findings from this study was the failure to detect significant evidence of oxidative stress via the measurement of MDA concentrations. The inability to detect increases in MDA is usually understood to indicate that there was no increase in oxidative stress, i.e., free radical formation. However, another question is brought to light. That is, are measurements of MDA alone a reliable biomarker of oxidative stress in exercise studies? A worthwhile endeavor would be to repeat this study.
using a second biomarker of oxidative stress (e.g., pentane levels) and/or more sophisticated techniques for detecting free radical formation (e.g., electron paramagnetic resonance spectroscopy). The use of a second confirmatory test and/or direct measurement of free radical formation would increase the value of the data. Such an analysis would also allow an assessment of the utility of MDA concentrations and its reliability in the detection of free radical formation, i.e., oxidative stress. Although the measurement of MDA has become a standard in free radical research, the detection of oxidative stress is undeniably more valuable when used in concert with a whole battery of oxidative damage tests.

The failure of MDA measurements to detect an increase in the generation of free radicals could indicate that these trained athletes did not have an increase in oxidative stress. As these runners have all been running a consistent number of miles for years, it is possible that they have very well developed enzymatic antioxidant defenses. Accordingly, there would not be a significant increase in MDA concentration after the 5K run. Therefore, it would be helpful to measure the actual antioxidant enzyme levels (e.g., SOD) as well as the MDA in testing this theory. In addition, it would be helpful to compare enzyme levels in sedentary vs active persons with and without beta-carotene supplementation. It has been supposed that the active person will require more antioxidant defenses due to the increase in oxygen consumption. However, this may not be the case in the well trained athlete as previously thought. On the contrary, it may be the sedentary individual with lesser defenses, i.e., poorly adapted antioxidant enzymatic mechanisms, that requires antioxidant
supplementation. Those persons embarking on a more active lifestyle may be the ones that are in particular need of non-enzymatic means of combating free radical formation. The detection of oxidative stress may be more prevalent in these individuals. Consequently, any beneficial advantage of beta-carotene may be more easily detected using sedentary subjects.

Another possibility concerning MDA is that the ability of beta-carotene to prevent free radical formation may not be detectable acutely in the active well-trained subject. It is possible that MDA levels will be lower with exercise following chronic supplementation and, as such, an increase in oxidative stress would only be detectable with consecutive days of running or longer endurance events (e.g., marathon). It may be that more intense activity is required to document a reduction in oxidative stress by beta-carotene.

This study did not measure actual serum beta-carotene concentrations. Information gleaned from the literature indicated that the chosen protocol would insure that the body would be saturated with beta-carotene. Regardless, it is possible that some of the subjects were not saturated resulting in a decrease in its antioxidant effects. If this were true, then a significant decrease in MDA would not be found. Furthermore, there may be subject variability in the absorption and storage of beta-carotene within the body. Some of the subjects may have more or lesser ability to absorb and/or store this vitamin. It might be helpful to determine intestinal absorption prior to the the study of its antioxidant effectiveness as well as total body stores.

There is the prospect that some individuals are “responders” to supplementation, while others are “nonresponders”. Certain
individuals may simply respond to the use of beta-carotene, while others do not. Only a large scale evaluation of beta-carotene ingestion would delineate this possibility. Likewise, some individuals may have varying rates of release of beta-carotene from storage sites. When the need of increased beta-carotene effects are present, some persons may allow release in a very expedient manner, while others may require the development of a new "receptor" or transport mechanism to allow its liberation. If release is a very slow process, then any acute effects may be undetectable with no perceptible improvement in parameters such as those measured in this study.

It was previously discussed that there is interaction between the antioxidant vitamins C and E. This is a known occurrence. At present, there is no data that reports on any beta-carotene interaction with any other antioxidant. This possibility does exist and may represent a previously unknown source of benefit or detriment in the activity of beta-carotene. This study did not undertake to determine the content of vitamin C or vitamin E in the diet or in the subjects themselves. However, even if no interaction does exist, the total body stores of these two antioxidants could play a role in combating free radicals and their resultant oxidative stress. Could some of these subjects have very high levels with concomitant maximizing of antioxidant effects by these vitamins? If these vitamins were at such high levels, could there be any detectable consequence of beta-carotene supplementation? This investigation does not provide answers to these questions. Further analysis of beta-carotene's antioxidants effects should consider this possibility with measurement of the concentration of vitamin C and vitamin E in the diet and in the body.
This study utilized only eleven subjects and, although most reports have used less subjects than this, more subjects would certainly increase the likelihood of discovering a plausible explanation of the proposed hypotheses. A follow-up study of thirty or more subjects would perhaps provide support to the proposed speculation. In addition, the evaluation could include both athletes and sedentary individuals, and compare the effects of training, of exercise duration, and severity of metabolic challenge.

In order to avoid any environmental influences, this study could be repeated using a treadmill for all the running data, or a bicycle ergometer using trained cyclists (collecting all the data in a climate controlled setting). However, climate controlled settings may disallow a potential source of detectable usefulness. Those who exercise at high altitude or in polluted air may show actual enhancement of performance by the use of beta-carotene due to its antioxidant properties. This is an area of potential investigation. In addition, the use of similar subjects within a narrow age range (e.g., 30-35 years old) would avoid the confounding factor of the aging process in older subjects.

A long-term study following runners for one to two years and monitoring their race performances with periodic measurements of MDA and VO_{2max} would account for any unforeseen variations in lifestyle, training, diet, etc. MDA concentrations may represent a very subtle biomarker. Ongoing and repetitive measurements would allow detection of meager, perhaps significant, changes. Moreover, prolonged monitoring of dietary consumption of all antioxidants would permit
analysis of antioxidant interactions or marked variations in consumption. These observations could also include determination of dietary fat intake. Beta-carotene is a fat-soluble substance and, as a consequence, its intestinal absorption is affected by fat intake. Any analysis of beta-carotene's capabilities would be influenced by fat within the diet. This aspect of beta-carotene was not determined in this study.

One interesting speculative thought concerns the potential of a psychological benefit of beta-carotene. Could beta-carotene affect neuronal synapses within the brain? Could this effect cause mood enhancement resulting in improved performance? There is no data to support this theory, yet it is known that even placebos may aid the body in various ways. This study was such that any placebo effect was theoretically negated by the double-blinded cross-over design. However, the possibility of a yet undiscovered psychological benefit does exist.

Another speculative suggestion involves the potential of any hormone-like action or other sympathetic stimulatory activity. Does beta-carotene have any epinephrine-like effects? If so, then this could perhaps explain the improved 5K performance with no significant effects on oxidative stress or VO_{2max}. Could beta-carotene have any stimulatory effects on the sympathetic nervous system? If so, then similar to any possible epinephrine-like effects, this could explain the failure to detect any decrease in oxidative stress or improvement in VO_{2max} while 5K race performance improved. Could beta-carotene serve in a synergistic capacity, interacting with some other substance, to result in ergogenic advantages? Could beta-carotene have some
unknown analgesic quality? If so, then athletes would be able to
withstand increased physical efforts, suffer less pain and discomfort,
and enjoy improved athletic ability in excess of their usual
capabilities. Does this vitamin A precursor have some undiscovered
ability to prevent fatigue, either physiologically or psychologically?
Could there be some ability to promote the healing of muscle tissue
during chronic exercise allowing for more intense training and, hence,
 Improved times in a 5K run? Is it possible that beta-carotene
influences local blood flow via reduction of ROS or some other
mechanism? While increased blood flow may not cause a rise in \( \text{VO}_{2\text{max}} \),
it might serve as an ergogenic aid via improved removal of waste
products of metabolism. Perhaps this could be measured via serum
lactate or ammonia levels (although the samples may be too dilute to
show significance). All of the above would go undetected by such
measures as MDA or \( \text{VO}_{2\text{max}} \). These questions do not have answers but
allude to areas of research which would further the work of the current
investigation.

Beta-carotene is but one of over 600 carotenoids found in
nature. Only about fifty (50) have been shown to possess provitamin A
activity. The most active, and often major, provitamin A carotenoid is
all-trans \( \beta \)-carotene. Could there be some type of intracellular
conversion to some other form following absorption resulting in
decreased antioxidant capacity? Could beta-carotene require some
conversion into another form within the cell to exhibit its antioxidant
properties? Are certain prerequisite intracellular conditions necessary
for this to occur? Is it possible that without this conversion, some
effect of beta-carotene will occur, while its antioxidant ability will be impaired? The limited available research did not undertake to study these possibilities.

Lastly, could beta-carotene aid oxygen delivery in the tissues or act as a membrane stabilizer unrelated to its ability as an antioxidant? Previous research has noted beta-carotene's ability in low oxygen pressures. However, might there be some ability to improve oxygen delivery deep within the active tissues which aid in performance? Could there be some effect on red blood cells and its hemoglobin's ability to carry and release oxygen? Does this provitamin interact with cell wall structures in such a way as to function as a membrane stabilizing supportive structure? Perhaps this would serve as a protective mechanism against organelle disruption, such as mitochondria, due to intense activity unrelated to free radical formation. Could beta-carotene have some ability to aid in the repair of cell membranes once they are damaged distinct from its antioxidant properties? All of these questions remain speculative and, as yet, unanswered.

Certainly, this investigation has been a productive undertaking. No studies were previously available to evaluate the antioxidant effects of beta-carotene alone. Some of the results obtained in this study are encouraging, however, much uncertainty persists in the final analysis. Although it remains to be seen what precise role beta-carotene plays in the body's fight against free radical damage, this data should serve as a template for continued investigation.
REFERENCES


15. Jenkins RR. Free Radical Chemistry: Relationship to Exercise. 


133. Bramich K, McNaughton L. The effects of two levels of ascorbic acid on muscular endurance, muscular strength and on VO$_{2\text{max}}$. *Int Clin Nutr Rev.* 7:5-9, 1987.


APPENDIX A

FOOD DIARY QUESTIONNAIRE
24-HOUR RECALL or FOOD DIARY

Please fill out the 24-hour dietary recall or food diary on three separate days.
Try to do it the same day of the week prior to beginning each phase of the study.
Pick days that make up the usual type of meals that you normally eat.
Follow the instructions as best you can.

This can be done one of two ways.
1) You can write down what you had the day before, or
2) You can write it down as you eat the food, or drink, etc.
   (I would prefer the second one!)

Write down the day of the week that you are writing down.
Also write down the date as well.
Do follow the number of the sheet as noted: 1st, 2nd, 3rd.
Principle

The purpose of the twenty-four-hour recall is to provide information on the respondent's exact food intake during the previous twenty-four-hour period or preceding day. Such information can be used to characterize the mean intake of a group. If habitual intakes of individuals are required, however, multiple replicate twenty-four-hour recalls must be used.

The twenty-four-hour recall is conducted in four stages using a standardized protocol. In the first stage, a complete list of all foods and beverages consumed during the previous twenty-four-hour period or preceding day is obtained. In the second stage, detailed descriptions of all the foods and beverages consumed, including cooking methods and brand names (if possible) are recorded, together with the time and place of consumption. In the third stage, estimates of the amounts of all foods and beverages consumed are obtained. Finally, in the fourth stage, the recall is reviewed to ensure that all items have been recorded correctly.

The advantages of the twenty-four-hour recall method include low respondent burden, high compliance, low cost, ease and speed of use, use of a standardized interview, an element of surprise (so that the respondent is less likely to modify his or her eating habits), and its suitability for illiterate respondents. Disadvantages include its reliance on memory, making it an unsatisfactory method for the elderly and for young children. Errors in the estimation of portion sizes of foods also occur, but can be reduced by using food models of various types to assist the respondent. Graduated food models are preferred because their use tends to prevent 'directed' responses, a phenomenon observed when simulated plastic food models representing 'average' portion sizes are used (Samuelson, 1970). The flat slope syndrome may be a problem in the twenty-four-hour recall method (Gersovitz et al., 1978): in this syndrome, individuals appear to overestimate low intakes and underestimate high intakes—sometimes referred to as 'talking a good diet'. Subjects completing single twenty-four-hour recalls are likely to omit foods which are infrequently consumed.

When conducting the interview, both interpersonal and technical skills are important: for example, the interview must always be conducted with an open and pleasant manner, with the aim of being friendly, diplomatic, empathetic, and determined, as appropriate.

If possible, the interview should be conducted somewhere quiet. The interviewer should start by establishing a rapport with the respondent, who should be told that questions will cover all the food and beverages consumed during the preceding day with emphasis on the pattern of eating. Stress that all responses will be confidential and emphasize the importance of providing the correct information. Avoid asking questions about specific meals (e.g. breakfast, lunch, supper) or about snacks. Avoid showing any signs of surprise, approval, or disapproval of the subject's eating pattern (i.e. be non-judgemental at all times). Respondents should not be told in advance that a twenty-four-hour recall will be conducted on a particular day's food intake to avoid any changes in the food intake of the subject.

The success of the twenty-four-hour recall depends on the subject's memory, the ability of the respondent to convey accurate estimates of portion sizes consumed, the degree of motivation of the respondent, and the skill and persistence of the interviewer (Acheson et al., 1980).

Stage 1: Recall of foods and drinks consumed

The recall interview should commence with the first food and/or drink consumed in the morning. The interviewer should use neutral questions such as:

I would like you to tell me what you had to eat or drink after you woke up yesterday morning. What was the time? Did you eat that food at home? What did you have next and when was that?

Proceed through the day, repeating these questions as necessary, and record each food or drink consumed in the appropriate column of the twenty-four-hour recall form.
Stage 2: Description of foods and drinks consumed

During this stage, the interviewer should go over each of the responses made by the respondent, probing for more specific descriptions of all the foods and drinks consumed, including cooking methods and (if possible) brand names. These details are recorded in the third and fourth columns of the form (Table 1.2). Information on the place and time of eating should also be obtained and recorded in the first two columns. For homemade composite dishes, the amount of each raw ingredient used in the recipe should be recorded, the number of serving sizes for the recipe, and the amount of the composite dish consumed by the subject.

Guidelines for appropriate prompts for specific food items are given below:

- **meat:**
  - kind of meat
  - description of cut
  - raw or cooked weight
  - method of cooking
  - lean or lean+fat
  - bone in or not (waste factor)

- **fish/sea food:**
  - kind of fish/sea food
  - raw or cooked weight
  - method of cooking
  - bones/skin/shell (waste factor)

- **poultry:**
  - kind of poultry
  - parts or pieces eaten (e.g. breast, thigh)
  - raw or cooked weight
  - method of cooking
  - white or dark meat
  - meat+skin or meat only
  - bones (waste factor)

- **fats:**
  - kind of fat
  - brand name (if possible)

- **milk products:**
  - kind of dairy product
  - brand name (if possible)
  - percentage fat (as butter fat or milk fat)

- **cheese:**
  - type (Edam, Swiss, cream, etc.)
  - percentage fat (if possible)

- **bread/rolls:**
  - type of grain (rye, whole-wheat, etc.)
  - homemade/bought
  - size: standard or unusual
  - toasted or not
  - topping/condiments

- **baked goods:**
  - type of product
  - whether iced or not
  - homemade or commercial
  - type of filling

- **cereal/pasta/rice:**
  - type of grain
  - brand name
  - raw or cooked weight
  - enriched or not
  - record: cereal + milk (if dry quantity unknown)

- **vegetables:**
  - fresh/frozen/canned
  - peeled/unpeeled
  - method of cooking
  - topping (butter, etc.)

- **fruits:**
  - fresh/frozen/canned
  - peeled/unpeeled
  - type of liquid (heavy, light)
  - sweetened/unsweetened

- **beverages/soup:**
  - volumetric or fluid ounces
  - size of can or bottle
  - fresh/frozen/canned/bottled
  - fruit juice: sweetened/unsweetened
  - added vitamins/minerals (e.g. Vit. C)
  - coffee: brewed, instant, decaffeinated, regular
  - soups: homemade/canned
  - soups: dilutant (milk/water)

- **take-out foods:**
  - restaurant name
  - food/beverage name
  - size of portion (small, medium, large)
  - condiments added

- **candies, etc.:**
  - brand name
  - size, price, or amount
Stage 3: Estimation of amounts

- Quantities can be recorded by the interviewer as volumes—milliliters, pints, cups, etc.; or as weights—grams, pounds, ounces, etc. Then convert all amounts to the equivalent number of grams.
- Use graduated food models (Fig. 1.1), such as those developed by Nutrition Canada (Health and Welfare Canada, 1973), to assist the respondent in estimating amounts. Display only appropriate models and emphasize that they are only a guide.
- Household cups, glasses, bowls and spoons, familiar to the respondent, can also be used to estimate amounts. If used, these should be calibrated.
- A ruler can be useful for estimating the thickness of slices of meat, cheese, and cake.
- Use counts for eggs and slices of bread.

Stage 4: Review of interview data

At the end of the interview, it is important that the interviewer reviews the recall to ensure that all the items have been recorded correctly. This can be accomplished using a statement such as the following:

*I will read back to you what I have recorded to make sure that I have not made any mistakes.*

Finally the respondent should be asked about the use of vitamin and mineral supplements, protein or diet drinks and also asked in a non-threatening manner about any alcohol consumed, e.g.

*Did you have any alcoholic drinks during the day?*

Enquire about anything consumed in the middle of the night. As a final check, the interviewer should scan the recall in case any food groups have been omitted and should politely enquire about any missing items (e.g. meat or milk). The interviewer should then ask the subject whether the day of the recall represented a 'normal' day. At the end of the interview, the interviewer should thank the respondent for his or her time and cooperation.

References


<table>
<thead>
<tr>
<th>Date</th>
<th>Day of the week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subject ID No.</td>
<td>Name of Subject</td>
</tr>
<tr>
<td>Place Eaten</td>
<td>Time</td>
</tr>
</tbody>
</table>

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APPENDIX B

RATINGS OF PERCEIVED EXERTION
Ratings of Perceived Exertion (RPE)

6
7...very, very light
8
9...very light
10
11...fairly light
12
13...somewhat hard
14
15...hard
16
17...very hard
18
19...very, very hard
20

5 min________
10 min________
15 min________
20 min________
End________
APPENDIX C
LETTERS OF PERMISSION
July 8, 1998

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Re: Permission to copy

Dear CRC Press,

I am presently a doctoral candidate at the Louisiana State University in Baton Rouge, La. In the department of Kinesiology. Although I already possess a Doctor of Medicine degree, I have been pursuing a Ph.D. degree in Exercise Physiology. At the present time, I am writing my dissertation which has prompted this correspondence.

My area of research involves the effects of beta-carotene on oxidative stress and exercise performance. Consequently, my dissertation will involve several references to free radicals and nutrition.

The purpose of this letter is to request permission to use one figure from the textbook entitled “Free Radical Mechanisms of Tissue Injury” (chapter 1). It is edited by Mary Treinen Moslen, PhD and Charles V. Smith, PhD. The copyright year is year 1992.

The figure to which I am referring is Figure 1 found on page 7.

Additionally, I intend to submit my data for publication at some later date. Therefore, at this time, I would also like these figures for this submission as well.

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The purpose of this letter is to request permission to use several figures from the textbook entitled "Advanced Nutrition and Human Metabolism". It is the second edition and is edited by James L. Griffin, Sareen S. Gropper, and Sara M. Hunt. The copyright year is 1995.

The figures to which I am referring are Figure 1.2 found on page 5 and Figure 3.12 found on page 64. These figures will be used in my dissertation only.

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May 22, 1998

CRC Press, Inc.
2000 Corporate Blvd., N.W.
Boca Raton, Florida 33431

Dear CRC Press,

I am presenting writing my dissertation for completion of my PhD degree at the Louisiana State University in Baton Rouge, La., in Exercise Physiology. My research area involves antioxidants, free radicals and exercise performance.

The purpose of this letter is to request permission to use figure 8 on page 196 from the text Nutrition in Exercise and Sport, 2nd edition. This figure is from Chapter 9 entitled "The Significance of Vitamin E and Free Radicals in Physical Exercise." The book is copyrighted in the year 1994.

Thank you in advance for your consideration.

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Dear Dr. LeBlanc:

Permission is hereby granted to include Figures 5.8, 10.5, and 10.6 from

Champe PC, Harvey RA. Lippincott’s Illustrated Reviews: Biochemistry. 1994; Philadelphia:
J.B. Lippincott: 68, 114

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I am presently a doctoral candidate at the Louisiana State University in Baton Rouge, La. in the department of Kinesiology. Although I already possess a Doctor of Medicine degree, I have been pursuing a Ph.D. degree in Exercise Physiology. At the present time, I am writing my dissertation which has prompted this correspondence.

My area of research involves the effects of beta-carotene on oxidative stress and exercise performance. Consequently, my dissertation will involve several references to free radicals and nutrition.

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The figure to which I am referring is Figure 33-9 found on page 502.

Additionally, I intend to submit my data for publication at some later date. Therefore, at this time I would also like to request permission to use this figure for this submission as well.

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July 6, 1998

Lori Barber
The American Journal of Clinical Nutrition
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The figure in question is from an article entitled "Action of Ascorbic Acid as a scavenger of active and stable oxygen radicals" by Etsuo Niki (Am J Clin Nutr 1991;54:1119S-248). Specifically, it is figure 6, regeneration of tocopherol from tocopheroxyl radical by ascorbic acid found on page 1122S.

Thank you for your attention and consideration in this matter.

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176
June 2, 1998

Dr. Kim Edward LeBlanc
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Breaux Bridge, LA 70517

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The figure to which I am referring is figure 1 (page 694) which appeared in the article entitled "Update on the biological characteristics of the antioxidant micronutrients: Vitamin C, vitamin E, and the carotenoids" (J Am Diet Assoc. 1996; 96:693-702). This article was written by: Cheryl L. Rock, Robert A. Jacob, and Phyllis E. Bowen.

Thank you in advance for your attention and consideration in this matter.

Sincerely,

Kim Edward LeBlanc, M.D., FACSM
Kim Edward LeBlanc is a practicing physician specializing in sports medicine and family practice. He is board certified in family practice and holds a Certificate of Added Qualifications in Sports Medicine. In addition, he serves as team physician for the University of Southwestern Louisiana (all sports), the Louisiana IceGators (professional ice hockey), and the Lafayette SwampCats (professional indoor soccer). He holds a clinical associate professor faculty appointment in the Department of Family Medicine at the Louisiana State University Medical Center in New Orleans and the University Medical Center in Lafayette, Louisiana.

Dr. LeBlanc is a Fellow of the American Academy of Family Physicians and a Fellow of the American College of Sports Medicine. He has published over a dozen articles in professional journals, authored chapters in various medical texts, and lectured both locally and nationally on sports medicine topics. He has participated in several research projects but has a keen interest in the use of antioxidants in the enhancement of exercise performance and disease prevention.

As a result of his interest in sports medicine, Dr. LeBlanc made the decision to expand his knowledge base and research skills by pursuing the Doctor of Philosophy degree in Kinesiology. His emphasis was in the branch of exercise physiology. He has accomplished this goal and will be awarded the degree of Doctor of Philosophy at the commencement exercises to be held on December 18, 1998.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Kim Edward LeBlanc

Major Field: Kinesiology

Title of Dissertation: Beta-Carotene and Exercise Performance: Effects on Race Performance, Oxidative Stress, and Maximal Oxygen Consumption

Approved:

[Signature]

Major Professor and Chairman

[Signature]

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signature]

[Signature]

[Signature]

[Signature]

Date of Examination:

7 October 1998