EFFECTS OF AGING AND PHYSICAL ACTIVITY ON SUPEROXIDE DISMUTASE IN HUMAN ERYTHROCYTES

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Abstract

This study investigated the relationship of the superoxide scavenging enzyme, superoxide dismutase (SOD), with respect to age, gender and physical activity levels of subjects. Blood was collected from 129 healthy human volunteers (80 males and 49 females) between the ages of 7 and 87. A physical activity index (in kcal/wk) was calculated for each subject.

A continuous line assay for Cu,Zn-SOD activity was performed and a function analogous to a Lineweaver-Burke equation in a nonclassical enzyme assay was used to derive a linear reciprocal plot, whose slope was proportional to (kcat Et/Km)'. This kinetic parameter is a reflection of total SOD activity in the RBC.

In order to isolate the influences, if any, of age, physical activity and gender on (kcat Et/Km)', subjects were divided into the following groups: (1) Active vs Inactive, (2) Male vs Female, (3) 20 year age brackets, and (4) 20 year age brackets subdivided into Active or Inactive individuals.

With advancing age there was seen to be a gradual decline in (kcat Et/Km)'. The drop in this parameter across the age spectrum was estimated to be approximately 50%. Physical activity, however, influences (kcat Et/Km)' in the opposite direction, as the active individuals displayed a higher mean (kcat Et/Km)' than their inactive counterparts. The mean value for the active group was 0.64 and for the inactive group it was 0.46 ($p<0.01$). A multivariate regression analysis indicated that the following linear function described the data:

(kcat Et/Km)' = -0.00188 * Age (years) + 0.000014 * Physical Activity (kcal/wk)

Hence, the decrease in (kcat Et/Km)' due to one additional year of life could be equally compensated for by an overall 134 kcal increase in weekly energy expenditure.

The experimental parameter, (kcat Et/Km)', seems to correlate with metabolic rate. This

assumption is supported by the following points: (1) SOD activity decreases by approximately 50% over a lifetime; this parallels a similar drop in metabolic rate, (2) This study showed that males had higher (kcat Et/Km)' values than females which agrees with differences in metabolic rates between the two sexes, (3) Active individuals were found to have higher (kcat Et/Km)' values than their inactive counterparts.

In this study it was also noted that extract protein concentration was higher in the active (6.57 ug/mL) versus inactive subjects (6.16 ug/mL) (difference in means was not statistically significant) and in the younger subjects as compared to the older ones. Presumably, oxidatively damaged proteins are more fragile and, therefore, cannot survive the harsh chloroform/ethanol extraction procedure used for partial purification of SOD. Hence, extract protein concentration is an index of cellular oxidative stress in which higher values are diagnostic of better health.

Arrhenius plots were used to resolve whether the higher SOD activities in young and active subjects was due to an increase in catalytic efficiency of the enzyme or changes in the amount of the SOD protein. No significant difference was detected between the Arrhenius plots of any of the subgroups studied. Thus, the increase in SOD activity was attributed to increased concentrations of the enzyme due to either enhanced gene expression or decreased SOD degradation rates.

DEDICATION

To my loving grandfather (Kashmir Manhas), my wonderful parents (Karam and Hardev Manhas), my brothers (Karan and Deepak) and my sisters (Sheila and Kiran) without whose support, encouragement and optimism this thesis would not have been possible.

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ABBREVIATIONS

ANOVA	analysis of variance
Arrhenius slope	-Ea
ATP	adenosine triphosphate
C	degrees Celsius
CAT	catalase
Ch/EtOH	chloroform/ethanol
cyt	cytochrome
dL	decilitres
Ea	energy of activation
ELISA	enzyme linked immunosorbent assay
EPR	electron paramagnetic resonance
ESR	electron spin resonance
Et	total amount of enzyme
GPx	glutathione peroxidase
GST	glutathione-S-transferase
Hb	hemoglobin
kcal/wk	kilocalories per week
kcat	enzyme rate constant
kDa	kilodaltons
Km	concentration of substrate which gives 1/2Vmax
LEP	lifespan energy potential
ln	natural log
LPO	lipid peroxides

M	molar = moles/litre
mL	millilitres
mM	millimolar = millimoles/litre
MLSP	maximum lifespan potential
MW	molecular weight
n	sample size
ng	nanograms
$O_2^{\cdot-}$	superoxide
PUFA	polyunsaturated fatty acids
R	universal gas constant
RBC	red blood cell
ROS	reactive oxygen species
[S]	substrate concentration
SDS	sodium dodecyl sulfate
SMR	specific metabolic rate
SOD	superoxide dismutase
SOD-1	copper, zinc-superoxide dismutase gene
TBARS	thiobarbituric acid reactive substances
TCA	tricarboxylic acid cycle
ug	micrograms
uL	microlitres
uM	micromolar = micromoles/litre
UV	ultraviolet
v	velocity

Vmax	maximal velocity
$V02_{max}$	maximal oxygen uptake

Literature Review

Introduction

Oxygen constitutes both a benefit and a threat to organisms that have taken advantage of an aerobic lifestyle. This is because it allows for efficient energy production, as aerobic oxidation of glucose provides fifteen times more energy than glucose's anaerobic conversion to lactate, while it becomes toxic to organisms when they are exposed to levels higher than they experience in their particular environmental niche (Fridovich, 1977a).

Throughout our planet's 5 billion year history, significant levels of atmospheric oxygen have only been present for the last 2.5 billion years at which time the first photosynthetic organisms began to appear (Gilbert, 1981). Prior to this time only infinitesimal levels of oxygen are believed to have existed in the earth's atmosphere (Frank, 1985). As the amount of ambient oxygen in the atmosphere slowly increased, this allowed for further evolution of plant and animal forms which moved towards progressively refined metabolic systems (Frank, 1985). Paralleling these changes must have been the evolution of biochemical defense mechanisms to protect the increasingly oxygen dependent organisms from the risk of excess oxidation of their cellular constituents. Thus, very early in the history of aerobic metabolism must have been the development of a homeostatic mechanism which monitored the levels of reactive oxygen species and protected against them (Haugaard, 1968; Fridovich, 1978; Gilbert, 1981). Aerobic organisms present today are probably the descendants of those primitive aerobic organisms which possessed the most efficient defense mechanisms against the toxic effects of oxygen (Frank, 1985).

Free radicals and other reactive oxygen species are produced during ordinary oxidative metabolism (Please refer to Appendix A for a definition of free radicals and Appendix B for an explanation of how free radicals arise from molecular oxygen). The deleterious actions of

these species, if not protected against, would destroy the cells in which they were produced (Fridovich, 1977b). Thus, the ability of organisms to live in an aerobic world, in which oxygen constitutes 21% of the earth's atmosphere, is completely attributable to an elaborate system of antioxidant defenses. This sentiment is eloquently expressed by Irwin Fridovich (Biology of Oxygen Radicals, *Am. Sci.*, **63,** 54, 1975):

> "All respiring organisms are caught in a cruel bind, in that the oxygen which supports their lives is a toxic substance in whose presence they survive only by virtue of an elaborate system of defenses."

The most important intermediate of oxygen metabolism is the superoxide radical and consequently a class of enzymes which have triggered a lot of interest are the superoxide dismutases (Please refer to Appendix C for a discussion of superoxide production in living systems). These enzymes afford the first line of antioxidant defense in all organisms.

In regard to superoxide dismutase (SOD), the erythrocyte is of particular importance since its principal function is the transport of oxygen and generation of $O_2^{\cdot-}$ is associated with autoxidation of oxyhemoglobin to methemoglobin (Kobayashi *et al.*, 1977).

The apparent ubiquity of SOD in oxygen-metabolizing organisms led McCord *et al.* (1971) to suggest that the prime physiological function of the enzyme is to protect the oxygen-metabolizer against the potentially detrimental effects of the superoxide free radical, and that the level of this enzyme, not catalase (a hydrogen peroxide scavenger), in an organism is the basis on which the organism can be classified as an aerobe, strict anaerobe, or aerotolerant anaerobe (Hatchikian and Le Gall, 1977). Strict anaerobes exhibit no SOD activity and generally no catalase (CAT) activity. Aerotolerant anaerobes, which do not use molecular oxygen as a terminal electron acceptor, but which can metabolize to a limited extent, are devoid of any CAT activity but do have SOD activity. All aerobes which reduce oxygen via cytochrome systems contain both activities. There are two possible explanations to account

for the presence of SOD and CAT in some strict anaerobes (Hatchikian and Le Gall, 1977): (1) Due to the photolysis of water that may have taken place under UV radiation before the establishment of the protective ozone layer in the atmosphere, some molecular oxygen was produced in the primitive oceans against which primitive strict anaerobes had to be protected or (2) SOD and CAT activities in strict anaerobes is a more recent acquisition allowing the survival of these organisms upon accidental contact with atmospheric oxygen.

Activity of Superoxide Dismutase

The role of SOD is to catalytically scavenge superoxide (Cudd and Fridovich, 1982; Halliwell, 1984). Superoxide dismutases react with superoxide at a diffusion-limited rate of 2×10^9 $M^{-}s^{-}$ at physiological pH (Rotilio *et al.*, 1972; Asada *et al.*, 1974). The reaction catalyzed by superoxide dismutase is the following (Fridovich, 1977a):

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \text{---> } H_2O_2 + O_2$$

This reaction is first order in enzyme and first order in the superoxide radical (Fridovich, 1977a).

Nonenzymatic dismutation occurs spontaneously at a rate of 1×10^5 $M^{-}s^{-}$ (McCord *et al.*, 1977; Frank, 1985), but the enzymatic reaction with superoxide dismutase is obviously much more effective in preventing excess buildup of superoxide in the cell, with a catalytic efficiency factor of 2×10^4 (McCord *et al.*, 1977). However, given that the superoxide dismutase concentration in a liver cell, for example, is greater than 10^{-5} M; whereas, the steady-state superoxide concentration is at least 4 or 5 orders of magnitude lower, the overall gain in the rate of the dismutation *in vivo* may exceed a factor of 10^9 (McCord *et al.*, 1977). The catalytic activity of superoxide dismutase with superoxide exceeds that of any other enzyme and its respective substrate. Rapid removal of superoxide is crucial because of the potential damage that can be initiated by superoxide itself. Even more important is the need

to prevent the formation of the extremely reactive hydroxyl free radical via an Fe^{+2} catalyzed Haber-Weiss reaction between superoxide and hydrogen peroxide:

$$2O_2^{\cdot-} + H_2O_2 \text{ ---> } OH^- + {}^{\cdot}OH + O_2$$

It appears that superoxide dismutases have evolved specifically to remove superoxide as they have not been found to act catalytically on any other substrate (Cudd and Fridovich, 1982; Halliwell, 1984). Hence, dismutation of superoxide is the true biological function of superoxide dismutases. Superoxide dismutase is an inducible enzyme and it appears that organisms living in elevated oxygen environments have increased levels of superoxide dismutase presumably to protect them against exposure to hyperbaric oxygen conditions (Gregory and Fridovich, 1973a and b).

Free Radical Theory of Oxygen Toxicity

The Free Radical Theory of Oxygen Toxicity postulates that the normal metabolic processes of aerobically respiring cells result in the production of a finite number of extremely reactive oxygen free radicals (Frank, 1985). Appendix D lists the important cytotoxic oxygen species which are formed during normal aerobic metabolic processes (Frank, 1985). Under basal metabolic and normobaric conditions these potentially cytotoxic species are adequately scavenged/controlled by a system of intracellular antioxidant defenses (Frank, 1985). Appendix E lists the major enzymatic and nonenzymatic antioxidant defenses which protect the cell against excessive oxidation of important cell components by reactive oxygen metabolites (Frank, 1985).

It is believed that oxygen toxicity occurs when the intracellular production of oxygen free radicals exceeds the capacity of the normal antioxidant defenses to detoxify them. When this normal oxidant-antioxidant equilibrium is disrupted such that the cell becomes inefficient in scavenging the reactive oxygen metabolites, uncontrolled oxygen free radical attack on

susceptible cell components ensues and oxygen toxicity is manifested (Frank, 1985).

It has been found that every major component of oxygen-metabolizing cells is susceptible to molecular and functional damage during hyperoxic exposure. Some of the important intracellular targets altered by oxygen free radicals are listed in Appendix F. Appendix G lists the vast array of pathologies in which oxygen free radicals are believed to play a role (Jenkins, 1988).

Free Radical Theory of Aging

An extension of the Free Radical Theory of Oxygen Toxicity is the Free Radical Theory of Aging. This theory was proposed by Denham Harman in 1956; however, it did not gain popularity until the discovery of the dismutation function of SOD by McCord and Fridovich (1969). Their discovery provided proof of a physiological role for free radical chemistry and breathed life into the Free Radical Theory of Aging. This theory implicates free radicals as being the modulators and perhaps initiators of the senescent process.

Two main postulates have emerged from the theory: (1) Cellular aging is attributable to a deterioration of cellular antioxidant defense mechanisms which remove the deleterious free radicals that are normal by-products of oxidative metabolism (Poot, 1991). This leads to elevated free radical levels and inevitable oxidative damage to vital cellular organelles (Barber and Brenheim, 1967), *or* (2) Cellular aging is the result of an accumulation of irreparable damage to the cells incurred by reactive oxygen species despite the activity of antioxidant defense mechanisms, which are not 100% efficient at scavenging oxygen free radicals (Poot, 1991). Therefore, low levels of free radicals are constantly present in the tissues. Consequently, over time there is an accumulation of damage (Lopez-Torres *et al.*, 1991).

Thus, either a breakdown of cellular defense mechanisms or the accumulation of irreversible damages induced by oxygen free radicals is believed to be responsible for many

of the changes which accompany aging. Hence, a central prediction of the Free Radical Theory of Aging is that there is an inverse relationship between an organism's antioxidant and enzymatic defense systems and the amount of free radical mediated damage (Sestini and Allsopp, 1991).

In order to be classified as a putative cause of aging, the process must satisfy the following criteria outlined by Strehler (1977): (1) universality, (2) intrinsicality, (3) progressiveness, and (4) deleteriousness. The principle of universality excludes accidental changes and those due to a genetic predisposition and intrinsicality bars environmental factors. However, the second criterion becomes somewhat problematic when one considers the close interplay between nutrients and metabolism. The third and fourth criteria are fairly obvious as it is evident that aging is a process in which the organism becomes increasingly vulnerable to external factors which heighten the body's susceptibility to potentially lethal stresses. In fact, aging has been described as the progressive accumulation of changes over time which are associated with an increasing susceptibility to carcinogens, drugs, diseases and environmental factors with advancing age (Harman, 1981; Stohs *et al.*, 1984). It is not surprising that free radical induced damage, which weakens the body through the devastating effects it has on all biologically important macromolecules, has been implicated in this process.

Furthermore, the importance to survival of oxidative detoxification mechanisms is well appreciated as it is firmly established that the efficiency of antioxidant defenses to detoxify reactive oxygen species is critical in maintaining an aerobic lifestyle (De *et al.*, 1991). Thus, aerobic organisms depend heavily upon the proper functioning and effectiveness of enzymes such as superoxide dismutase, glutathione peroxidase and catalase. Moreover, it appears that the balance of activities among these three enzymes controls the amount of toxic oxygen induced damage *in vivo* (Mailer *et al.*, 1991). Hence, a decline in antioxidative enzymatic

activity or a disruption of the delicate balance of the enzyme activities will seriously jeopardize the body with respect to cellular and mitochondrial damage. In fact, lipid peroxidation and eventual cell death are widely accepted as being the consequences of reduced endogenous antioxidant protection (Mailer *et al.*, 1991).

Although research has failed to unambiguously support a breakdown of cellular antioxidant systems, there is evidence that even minor depletions may be significantly damaging. For example, small decreases in cellular glutathione as a result of exposure to a model lipophilic peroxide were shown to lead to significant declines in DNA and protein synthesis (Poot, 1991).

The Free Radical Theory of Aging has gained support from many observations which have indicated that oxidative damage plays an integral part in the aging process. In fact, the Free Radical Theory of Aging, based on the astronomical amount of research it has generated, is currently one of the most popular theories of aging (Poot, 1991).

It is now well documented that the deterioration of membrane structures by free radicals and peroxide mediated processes is an important part of aging (Mailer *et al.*, 1991). Furthermore, longer lived individuals and/or species have been shown to have more effective defense mechanisms, in terms of quantitative differences, over their lifespans (Sohal, 1988; Wadhwa *et al.*, 1988). However, it should be noted that the supplementation of culture mediums with antioxidants has failed to significantly extend the lifespan of cultured diploid somatic cells (Poot, 1991). Such evidence suggests that the level of cellular antioxidants may act as a modulator rather than as a primary determinant of cellular aging in culture.

Nevertheless, based on the plethora of effects produced by oxygen free radicals, evidence for a role of these species in aging has been actively sought (Poot, 1991). Oxygen-free radical mediated damage includes lipid peroxidation (Freeman and Crapo, 1982), modifications of

DNA bases (Aruoma *et al.*, 1989a and b), impaired DNA replication (Poot *et al.*, 1988), single-strand breaks (Melho-Filho and Menighini, 1984), release of bases (Yamane *et al.*, 1987), mutagenesis (Hsie *et al.*, 1986), decreases in membrane fluidity (Hegner, 1980), elevated sensitivity to oxidant stress and osmotic fragility (Bartozs, 1981; Rifkind *et al.*, 1983), changes in enzyme activities (Stohs *et al.*, 1984; Pfeffer and Swislocki, 1982), and the formation of cytotoxic aldehyde compounds which have been implicated in protein cross-linking reactions (Gutteridge *et al.*, 1986). Perhaps the most important effect of free radicals which contributes to senescence is lipid peroxidation. Lipid peroxides are believed to be a significant factor in the pathogenesis of atherosclerotic cardiovascular disease in the elderly (Niwa *et al.*, 1989), a leading cause of morbidity and mortality in aged individuals.

Although many studies have been devoted to the hypothesis that free radical damage is associated with aging and as a result many positive arguments have emerged (Tappel *et al.*, 1974; Kimball *et al.*, 1976; Nohl *et al.*, 1979), it is nonetheless difficult to determine to what extent the effects of free radicals can account for the various changes witnessed in cellular aging (Somville *et al.*, 1985).

For one thing, the reaction times of free radicals are so short that linking the Free Radical Theory to certain diseases or aging based on analyses of free radical species in biological systems has been problematic (Hiramatsu *et al.*, 1992). Also, difficulties arise in evaluating this hypothesis because of the diversity of test systems examined and a lack of clarity as to what constitutes a valid and satisfactory test of the hypothesis (Poot, 1991). Furthermore, problems arise when one considers that cells have elaborated a variety of protective mechanisms (both enzymatic and nonenzymatic) in defense against the action of free radicals and products of their decomposition (Jozwiak and Jasnowska, 1985; Somville *et al.*, 1985). These include low molecular weight compounds found in the cytoplasm and plasma

membranes as well as specific antioxidant enzymes (Burton *et al.*, 1983; Sies and Cadenas, 1983; Smith and Lawing, 1983). It has been suggested that all protective processes of this type found in cells and involving antioxidants may be potential determinants of longevity in organisms (Jozwiak and Jasnowska, 1985). Moreover, it is believed that in mammals, the main source of endogenous free radical reactions may be initiated from oxygen (Harman, 1981).

In erythrocytes, the primary defense mechanisms employed against oxygen toxicity are the enzymes superoxide dismutase, glutathione peroxidase and catalase (Jozwiak and Jasnowska, 1985). Superoxide dismutase is one of the most important protective enzymes responsible for the removal of the deleterious products of oxygen metabolism and is present in large amounts in all aerobic cells (Fridovich, 1975).

It is conceivable that the progressive accumulation of damage with age due to free radicals may lead to disturbances in critical life processes, which in turn, alter proper cell functioning and have devastating effects on the organism (Jozwiak and Jasnowska, 1985). There is evidence that changes in superoxide dismutase activity relative to that of hydrogen peroxide consuming enzymes (i.e., glutathione peroxidase and catalase) have deleterious effects (Pigeolet *et al.*, 1989). Thus, under normal conditions, a fine balance may exist between the rate of hydrogen peroxide formation via dismutation of superoxide and its removal by glutathione peroxidase and catalase (Hiramatsu *et al.*, 1992). Decreased activity of superoxide dismutase will lead to elevated levels of superoxide; whereas, increased activity of superoxide dismutase will lead to an increase in the steady state levels of hydrogen peroxide (Hiramatsu *et al.*, 1992). Both hydrogen peroxide and superoxide can initiate further radical reactions via the formation of the extremely reactive hydroxyl radical and singlet oxygen. These reek havoc on biological systems. Norris and Hornsby (1990), who transfected an expression vector

containing a superoxide dismutase gene into adrenocortical cells, reported that this led to cell necrosis and nuclear fragmentation. This effect may be ascribed to enhanced hydrogen peroxide and hydroxyl radical formation due to elevated superoxide dismutase activity (Yarom *et al.*, 1988). Alterations of antioxidant enzymes is not difficult to imagine. These changes may be brought about due to alterations in protein synthetic machinery (Rattan, 1991; Eleftheriou *et al.*, 1991) or due to modifications of the proteins themselves (Kristal and Yu, 1992). It has been shown that cellular aging, both *in vivo* and *in vitro,* is accompanied by changes in the synthesis of several proteins (Eleftheriou *et al*, 1991). These changes are both quantitative and structural and have been reported for intracellular, plasma membrane, and extracellular (secreted) proteins (Maciera-Coelho, 1988). However, the area of protein synthesis has received the most attention. Extensive studies in this area have revealed that the protein synthetic activity of tissues from a variety of organisms ranging from fungi to humans, declines with advancing age (Richardson and Semsei, 1987). A related decline in transcription is also generally observed in tissues (Richardson and Semsei, 1987). Studies on various components of the protein synthetic machinery during cellular aging indicate a loss in the efficiency and accuracy of ribosomes, an increase in the levels or rRNA and tRNA, and a decrease in the amounts and activities of elongation factors (Rattan, 1992). Furthermore, it must be realized that the antioxidative enzymes that protect biological macromolecules from free radical attack, are also targets of free radicals and modification by glycation. *In vitro* studies by Arai's group (1987) who investigated the effects of glycation on erythrocyte Cu,Zn-SOD indicated inactivation of this enzyme via such a modification.

Protein modifications with respect to enzyme activities, thermostability and immunoreactivity have also been documented in aging cells (Somville *et al.*, 1985). Glucose-6-phosphate dehydrogenase is probably the best studied enzyme in this regards (Holliday and

6-phosphate dehydrogenase is probably the best studied enzyme in this regards (Holliday and Tarrant, 1972; Duncan *et al.*, 1977; Houben *et al.*, 1984a and b). Enzymatic modifications with age have also been observed for 6-phosphogluconate dehydrogenase (Holliday and Terrant, 1972), hypoxanthine-guanine phosphoribosyl-transferase (Goldstein and Moerman, 1975), enolase (Sharma *et al.*, 1976), aldolase (Steinhagen-Thiessen and Hilz, 1976), lactase dehydrogenase (Lewis and Terrant, 1972), glutathione reductase (Harding, 1973), isocitrate lyase (Reiss and Rothstein, 1975), glucose-6-phosphatase (Grinna and Barber, 1975) and superoxide dismutase (Reiss and Gershon 1976a and b). A popular interpretation of these enzyme modifications is that a post-translational effect on the enzyme induces a conformational change (Dreyfus *et al.*, 1978; Rothstein, 1981). For example, the molecular mechanism of glucose-6-phosphate dehydrogenase alteration has been shown to be a shift in the equilibrium between the active dimer and an inactive monomer with the altered enzyme acting as an intermediary (Houben *et al.*, 1984).

Such alterations may occur in antioxidant enzymes leading to devastating results. These putative changes would effect every system of the body. At present, the Free Radical Theory of Aging is championed by many members of the scientific community as several areas of research lend support to the central hypotheses of the theory (Please refer to Appendix H for further details).

Superoxide Dismutase and Aging

Free radical reactions may play a pivotal role in contributing to organismal senescence by causing partly irreversible damage. Hence, cellular SOD levels may constitute an important factor in influencing the rate of senescence.

Although there has been a considerable amount of research conducted in an attempt to elucidate changes in antioxidant enzymes with advancing age, no real consensus seems to

have been reached. One of the most intensely studied antioxidant enzymes in relation to the aging process is, undoubtedly, SOD. Researchers examining this enzyme with respect to age have reported increases, decreases and no change in SOD activity between young and old individuals.

These conflicting results are likely due to differences in assay methods, sample preparations, populations studied, conditions of assays, definition or normalization of specific activity, age classifications, animal models employed, gender and animal tissue studied. Using the xanthine/xanthine oxidase/cytochrome c method to assay SOD activity, Niwa *et al.* (1988) found no change in skin SOD levels, reported as SOD activity per milligram total protein, of healthy older individuals. However, these researchers noted a diminished capacity for SOD induction under conditions of oxidative stress in the aged individuals.

Again using the xanthine/xanthine oxidase/cytochrome c method to assay for SOD, Niwa *et al.* in 1989 determined SOD activity levels in blood cell populations of old individuals (65+ years of age) and found them to be comparable to young adult controls (18-48 years of age). SOD activity was reported as units per milligram of total protein.

Joenje *et al.* (1978) assayed SOD both immunochemically by a radial immunodiffusion technique and by enzymatic activity via the xanthine/xanthine oxidase/nitroblue tetrazolium method in erythrocytes of human donors aged 1-98 years old. No change was observed in enzyme activity per unit enzyme antigen as a function of donor age. Although these investigators attempted to detect changes in the *specific activity* of SOD as a function of age, their study was limited in that they only immunochemically assayed SOD in four young subjects (mean age 5 years) and four old subjects (mean age 64 years). Thus, their sample size was quite small. There may even be problems in comparing such young children to the older age group in that it has been reported that SOD activities increase until young adulthood.

Another flaw in the experimental design was Joenje *et al.*'s referencing of SOD levels to hemoglobin content of the hemolysates, a parameter which itself is not constant with respect to age.

SOD activity was assayed using the autoxidation of epinephrine method by Stevens *et al.* (1975). They were unable to show any change in Cu,Zn-SOD activity per milligram non-hemoglobin protein in human erythrocytes as a function of donor age.

Similarly, Kellog III and Fridovich (1976) have reported no change in Cu,Zn-SOD activity in rat liver with age. Also, no change in the activity of Cu,Zn-SOD in the cortex, striatum and hypothalamus of aged mice has been reported by Danh *et al.* (1983).

Using the autoxidation of pyrogallol method to assay for SOD, De *et al.* (1991) found no change in SOD activity reported as units per milligram total protein in hepatic mitochondria.

A recent, very comprehensive study was conducted by Ceballos-Picot *et al.* (1992). To determine whether SOD in human erythrocytes is altered as a function of age they measured Cu,Zn-SOD activity in 167 healthy subjects aged one month to 63 years of age. These subjects were classified into five age categories: Group 1 (newborn-age one), Group 2 (1-11 years), Group 3 (11-25 years), Group 4 (25-40 years), and Group 5 (40-63 years). SOD activity was determined by monitoring the autoxidation of pyrogallol and reported as units per milligram of hemoglobin. The results showed that the mean SOD activity in erythrocytes was comparable in groups one and two, but was significantly higher than in the other three groups. Moreover, there was no significant difference between mean SOD activities among groups three, four or five. However, the mean activity levels of these groups were markedly lower than that of groups one and two. Perhaps if these researchers had extended their study to include more aged individuals (i.e. those 65+ years of age) they would have witnessed a

similar decline in SOD activity levels in this group as compared to the other groups studied.

Jozwiak and Jasnowska (1985) studied changes in SOD levels with age using two different methods of SOD activity determination and three age categorizations. The first method employed was the adrenaline/adrenochrome system for SOD determination and the second was the riboflavin/methionine/nitroblue tetrazolium method. Subjects were classified as young (4-14 years of age), middle/mature (25-50 years of age) or senescent (65-80 years of age). These investigators found that according to both methods of determination, SOD activity decreases slightly in human erythrocytes with advancing age of donors. However, the decline was not statistically significant. SOD activity was measured as units of activity per gram of hemoglobin.

In contrast to the many reports that SOD activity levels remain unchanged with advancing age are several studies indicating loss of SOD activity with aging.

Reiss and Gershon (1976) compared the "specific activity" of cytoplasmic Cu,Zn-SOD (defined as units of SOD activity per gram hemoglobin) in homogenates of liver, brain and heart of aging rats and mice. Note that these researchers were not truly measuring *specific activity* of SOD as they did not determine amount of SOD protein, but rather expressed SOD activity levels in terms of hemoglobin content. SOD activity was determined using the pyrogallol method and in liver it was found to decrease 2.5 fold as a function of age. This phenomenon was ascribed to a decrease in SOD activity per unit antigen. In contrast, SOD activity exhibited only a marginal decline in heart and no change in brain.

Despite the differences observed in the age-related alterations in SOD "specific activity" in homogenates of the different organs, the enzyme exhibited a dramatic decline in catalytic activity per antigenic unit in all three organs with respect to advancing age in rats and mice (Reiss and Gershon, 1976).

These observations were the first to show that an accumulation of antigenically cross-reacting material with reduced catalytic activity in aging animals may not necessarily correlate with a decrease in "specific activity" of the enzyme in tissue homogenates. The findings of Reiss and Gershon (1976) indicate that the cells in old organisms are synthesizing more SOD molecules in order to compensate for the partial loss of enzyme activity in an attempt to maintain a constant SOD activity level.

Reiss and Gershon's observations are supported by the work of Ischiropoulous *et al.* (1990) who examined pulmonary SOD and reported that total SOD activity remains constant with age, but the proportion of relatively inactive SOD increases with age.

In 1981 Glass and Gershon found a decline in Cu,Zn-SOD activity with respect to erythrocyte age. Based on evidence that there is a loss of activity in many erythrocyte enzymes with advancing cell age (see Beutler, 1979 for a review), Glass and Gershon decided to study changes in SOD with respect to erythrocyte age as well as donor age. Hence, they separated erythrocytes into age-associated fractions by discontinuous density gradient centrifugation. This is possible because of the loss of cellular volume and increase in cellular density which accompanies erythrocyte aging.

Using the autoxidation of pyrogallol to determine SOD activity Glass and Gershon, in an elegant study, found a decrease in "specific activity" (reported as units per gram hemoglobin) of rat erythrocyte SOD with increasing cell age and also with increasing donor age. A striking discovery was that young erythrocytes of old animals exhibited considerably reduced SOD activity even when compared to old cells of young animals.

Concurrent with a loss of enzyme activity, Glass and Gershon observed an increase in functionally impaired enzyme molecules with increasing cell and animal age. These molecules antigenically cross-reacted with fully active molecules, but displayed either complete or partial

loss of catalytic activity per unit enzyme antigen. The average loss of activity was found to be 37% when cell fractions from old donors were compared with fractions of equivalent age from young donors. Somville *et al.* (1985) using immunotitration also demonstrated altered SOD molecules in human fibroblasts with increasing age of donors. They defined altered Cu,Zn-SOD molecules as those enzymes which display changes in thermostability (i.e. an accelerated denaturation rate at 70 degrees Celsius). These changes have been observed in the cytoplasmic SOD, but not the mitochondrial SOD of aging cells. Altered SOD was associated with the appearance of thermolabile tetramers of molecular weight 64 000 as compared to pure Cu,Zn-SOD which is dimeric and has a molecular weight of 32 000. The altered SOD molecules were evidenced as Cu,Zn-SOD tetramers and were observed only in the cytoplasmic fractions of old cells.

Interestingly, the altered SOD from old cells was found to disappear if it was incubated with cytoplasm from young cells. Conversely, cytoplasm from old cells induced the alteration of normal SOD isolated from young cells. Moreover, the young cytoplasm was "dominant" to the old cytoplasm in that the presence of young cytoplasm prevented the formation of Cu,Zn-SOD tetramers. Somville *et al. (1985)* speculate that a post-translational modification accounts for the alteration of Cu,Zn-SOD in old cells.

Another striking observation was the finding that only cytoplasmic SOD was significantly affected in old cells and mitochondrial SOD was not. This finding reinforces previous results of Houben and Remacle (1973) who reported that enzymes located inside organelles were not affected by aging while cytoplasmic enzymes were noticeably affected.

Umeki *et al.* (1987) have determined erythrocyte Cu,Zn-SOD activity levels using the xanthine/xanthine oxidase/nitroblue tetrazolium method. Reported as units of SOD activity per milligram of protein in blood SOD activity levels were found to decrease slightly in older

healthy individuals (mean age 59.9 years) as compared to younger healthy subjects (mean age 28 years). In addition, superoxide anion concentrations were significantly greater in the older group as compared to the younger one. Similarly, Im and Hoopes (1984) have claimed that Cu,Zn-SOD enzyme activity decreases with advancing age. Geremia *et al.* (1990) have also reported a decline in Cu,Zn-SOD activity in neuronal and glial cells of the aged rat cortex.

Comparatively fewer reports have indicated an increase in SOD activity with age. Nonetheless, some recent reports employing very sophisticated methods of analyzing the effect of elevated SOD levels have implicated increased levels of SOD in the aging process.

Del Maestro and McDonald (1987) reported an increase in SOD in rat brain with age. Also, total SOD in whole brain of aged rats was found to be increased by Mavelli *et al.* (1978). Confirming these results are the findings of Hiramatsu *et al.* (1992) which indicate elevated levels of cytosolic and mitochondrial SOD in aged rat brain as compared to those of younger adult brains.

Similar increases have been observed in other model systems. Sestini and Allsopp (1991) examined aging changes in *Drosophila melanogaster* flies and found an increase in SOD activity with age. In fact, an inverse relationship was observed between SOD activity and longevity. That is, the highest levels of SOD activity were measured in the shortest lived group, vestigial winged males; whereas the longest lived group, wildtype females, exhibited the lowest levels of SOD activities. SOD activity was measured via the inhibition of cytochrome c reductase method and it was reported as units of enzyme per milligram of total protein.

Sestini and Allsopp propose that the inverse relationship between SOD activity levels and longevity is due to either an enhanced rate of free radical production or an age-related decrease in the organism's defense system which leads ultimately to elevated free radical

induced damage.

Supporting this hypothesis, Sawada and Carlson (1990), found an increase in superoxide radical which appeared to be negatively correlated with survival in short-lived rotifer, *Asplanchna brightwelli.*

Some very interesting results have been obtained regarding the effects of excess SOD using genetic engineering techniques in which transgenic mice are produced which carry the human Cu,Zn-SOD gene. In this way, Cu,Zn-SOD gene dosage effects can be investigated. The locus for the Cu,Zn-SOD gene is the 21q22 segment of chromosome 21. This region contains as many as 100 other genes besides that coding for Cu,Zn-SOD, of which only 25 have been identified and mapped (Cooper and Hall, 1988).

Down's syndrome patients also present another useful model for examining the consequences of carrying an extra gene for Cu,Zn-SOD. Down's syndrome is a disease in which patients display an acceleration of age-dependent changes (Avraham *et al.*, 1991). These patients are characterized by trisomy 21 (that is they have 46 chromosomes plus one additional chromosome 21). However, approximately 5% of Down's syndrome patients, although clinically similar to trisomy 21 patients, have only a portion of chromosome 21 present in triplicate. This portion of chromosome 21, which has been identified as the 21q22 segment, is translocated to another chromosome (Summit, 1981).

Avraham *et al.* (1988) have studied the tongue muscle neuromuscular junctions from human Cu,Zn-SOD transgenic mice. These animals exhibited pathological changes indicative of premature aging. Other researchers have shown that the morphological changes in tongue muscle neuromuscular junctions of young adult transgenic mice are identical to those seen in the hindlimb muscles of aging mice and rats (Cardasis, 1983).

A subsequent study by Avraham *et al.* (1991) again looked at the aging of neuromuscular

junctions in human Cu,Zn-SOD transgenic mice in an attempt to test the hypothesis that overexpression of Cu,Zn-SOD is associated with premature aging.

In order to examine the aging process, several well-established age-dependent parameters were analyzed. Specifically, nerve terminal length, number of nerve terminal branching points and incidence of sprouting were examined. All of these processes are known to increase with advanced age in mice.

Avraham *et al.* (1991) found an acceleration of the age-dependent changes manifest in the morphology of the neuromuscular junctions of hindlimb muscles. Not only was there an acceleration of the age-dependent parameters, but also these changes were expressed earlier (i.e., at a younger age) in the human Cu,Zn-SOD transgenic mice as compared to their nontransgenic littermates. Avraham *et al.* considered this to be strong evidence that Cu,Zn-SOD overproduction is associated with premature aging of the nervous system.

Ceballos-Picot *et al.* (1991) using a similar experimental design have also investigated neurological changes in human Cu,Zn-SOD transgenic mice. They believe that excess Cu,Zn-SOD, by accelerating H_2O_2 generation, promotes oxidative damage in trisomy 21 cells and this is responsible for the neurological abnormalities characteristic of premature aging in Down's syndrome patients.

These researchers analyzed mice brain sections immunohistochemically and by *in situ* hybridization analysis. Their examination revealed that the Cu,Zn-SOD protein and mRNA are preferentially localized and expressed in the neurons. In fact, in the transgenic mice the activity of Cu,Zn-SOD was found to have increased 1.93 fold. Furthermore, a rise in brain lipid peroxidation measured by malondialdehyde concentration was evidenced. Ceballos-Picot *et al.* claim that these results support the hypothesis that a Cu,Zn-SOD gene dosage effect may play a primary role in the pathogenesis and accelerated aging features of Down's syndrome

brains.

Noteworthy, is the observation that loss of neurons in the aging, Down's syndrome and Alzheimer's disease brains is selective and follows the same pattern (Wisniewski *et al.*, 1985). Disruption of the regulation of the cellular antioxidant system is often deemed to be responsible for this selective attrition.

In support of this notion, it has recently been demonstrated that there is a preferential localization of Cu,Zn-SOD protein and mRNA in the vulnerable pyramidal neurons of Alzheimer's patients (Ceballos-Picot *et al.*, 1991).

The SOD activity in the cerebrospinal fluid of human subjects was determined by Hiramatsu *et al.* (1992) by electron spin resonance spectroscopy using the spin trap method. SOD activity levels in rat brains was also examined. In all cases SOD activity was assayed via the xanthine/xanthine oxidase/cytochrome c method. Cu,Zn-SOD activity in the cerebrospinal fluid of human elderly subjects and in all dissected brain parts of aged rats was found to be increased compared to younger counterparts. These results are supported by the findings of Bracco *et al.* (1991) who analyzed Cu,Zn-SOD activity in human cerebrospinal fluid using the polarographic method of catalytic currents and reported an increase in enzyme activity with age. Hiramatsu *et al.* suggest that the increase in SOD may be the expression of a self-protective mechanism employed by aging individuals to protect the brain against the rise in superoxide generated radicals which normally accompanies aging.

High levels of Cu,Zn-SOD have been implicated in the age-related process of cellular membrane deterioration (Balazs and Brooksbank, 1985; Groner *et al.* 1986). Furthermore, several experiments have shown that the physiological consequence of elevated Cu,Zn-SOD activity is accelerated cellular lipid peroxidation (Elroy-Stein *et al.*, 1986; Elroy-Stein and Groner, 1988; Schickler *et al.*, 1989). This, in turn, is believed to lead to damage of various

cellular constituents.

Exercise and Aging

In the laboratory setting, the best method of assessing overall cardiovascular/aerobic fitness is the measurement of VO_{2max} (or maximal oxygen uptake) during bicycle or treadmill exercise (Rowell, 1974). This technique is very effective, highly reproducible and relatively noninvasive. An individual's $VO_{2\ max}$ remains constant provided that he/she is exercising at least 50-60% of his/her muscle mass (Fleg and Goldberg, 1990).

$VO_{2\ max}$ is defined as the product of cardiac output and systemic arteriovenous oxygen difference and is a measurement of the cardiovascular system's maximal ability to deliver oxygenated blood to the body's periphery and the capacity of exercising muscles and other aerobic tissues to extract oxygen from the blood.

Gerontological research has consistently found that there is approximately a 10% declines in $VO_{2\ max}$ per decade beginning at 25 years of age. There is, however, evidence that regular aerobic exercise training attenuates the progressive decrease in $VO_{2\ max}$ during normal aging.

Heath *et al.* (1981) have reported a decelerated loss of $VO_{2\ max}$ with age in endurance-trained male athletes who were matched for training habits and performance in the same event at a similar age in order to estimate decline in $VO_{2\ max}$ in the elderly subjects by extrapolating $VO_{2\ max}$ values of younger counterparts. The loss in $VO_{2\ max}$ in the endurance trained older athletes was 5% per decade as compared to the 10% per decade decline witnessed in the sedentary population (Dehn and Bruce, 1972). This halving of the loss in VO_{2max} per decade suggested that the age-dependent decline in maximal aerobic capacity can be slowed down by maintaining a regime of optimal physical activity.

Improvements are also observed in per cent body fat, plasma blood lipids, glucose intolerance and insulin sensitivity in older subjects who train at high work intensities (Seal *et*

al., 1984). These are all physiological parameters which are known to worsen with age.

Data has also been collected which indicates that physical conditioning and maintenance of low body fat may prevent some of the age-related deterioration in lipoprotein lipid metabolism (Seals *et al.*, 1984). In fact, many studies have demonstrated that regular physical activity attenuates many of the physiological changes associated with aging. These include augmented maximal oxygen capacity, greater strength, lower plasma LDL cholesterol, better glucose tolerance and denser bones (Fleg and Goldberg, 1990).

Researchers investigating exercise and aging have generally found that many of the declines in cardiovascular and metabolic functions associated with advancing age are mediated to a large extent by age-associated decreases in physical activity and the accompanying changes in body composition.

In conflict with the benefits of exercise generally observed in the elderly, a study by Sohal (1991) reported a negative correlation between physical activity and longevity in *Drosophila* flies. Sohal found a two fold increase in both average and maximum lifespan of flies as a consequence of the elimination of flying activity.

Drosophila flies are very active and display a 100 fold increase in their rate of oxygen consumption when flying as compared to sitting. Thus, Sohal was able to control the metabolic rate of the flies by manipulating their flying activity. Low activity (LA) flies were those placed in solitary confinement under conditions preventing flight. Due to space restriction these flies could walk, but could not fly. The high activity (HA) flies, on the other hand, were housed in large cases which accommodated 200 flies and allowed ample space for flight.

The average and maximum lifespans of the HA flies were 19 ± 6 days and 40 days respectively; whereas those of LA flies were 44 ± 14 and 77 days respectively.

In addition, Sohal found that the mitochondria from HA flies exhibited a 39% higher rate of H_2O_2 production than the mitochondria of LA flies. Sohal attributed this to the higher rate of physical activity and oxygen consumption which he suggests resulted in a corresponding increase in prooxidant generation and peroxidative damage to mitochondrial membranes.

Nonetheless, the association between regular exercise and an attenuation of age-related physiological declines should not be ignored as it may present major public health implications for the elderly.

This thesis is based on the assumption that exercise levels of subjects will influence changes in the antioxidant enzyme, Cu,Zn-SOD with age. Little research to date has been conducted to elucidate the relationship between antioxidant status and exercise as will become apparent in the following section.

Exercise Induced Oxidative Damage

During exercise, various organs experience an increase in oxygen flux and therefore with physical training there is a chronic intermittent increased exposure to oxygen. Strenuous and exhaustive exercise as well as unaccustomed exercise have been reported to induce oxidative stress resulting in muscle injury (Meydani and Evans, 1993; Ji and Fu, 1992; Ohno *et al.*, 1992). Witt *et al.* (1992) were the first to report oxidative damage to proteins in exercised animals. It is reasonable to assume that the rate of free radical production is a function of oxygen flow through organ and muscle tissue (Kretzschmar and Muller, 1993). Indices of oxidative injury such as tissue lipid peroxidation, reversible enzyme inactivation (Fridovich, 1986) and cellular antioxidant systems have supported this claim (Ji *et al.*, 1992). The presence of abnormal protein carbonyls (derived from metal catalyzed oxidations of protein side chain amino acids) has also been used as an index of oxidative damage (Witt *et al.*

1992). Furthermore, *in vivo* and *in vitro* animal and human studies have provided direct and indirect evidence of free radical generation during and following exercise (Meydani and Evans, 1993). Direct evidence that exercise generates free radicals comes from the observation of increased electron paramagnetic resonance (EPR) and electron spin resonance (ESR) signals from exercised muscles (Kumar *et al.*, 1992). Indirect evidence comes from studies like that of Jenkins (1988) who found that acute exercise in rats led to an increased production of radicals evidenced by a uniform transient increase in lipid peroxidation.

In addition, physical training has been shown to enhance microsomal cytochrome p450 drug metabolism in humans (Frenkl *et al.*, 1980). Cytochrome p450 has both a monoxygenase activity and an oxidase function. The later is responsible for the liberation of superoxide and hydrogen peroxide. Meydani and Evans (1993) report that exercise influences oxidative metabolism and leads to the production of oxygen species which alter the membrane fatty acid composition, thereby increasing permeability and leakage of enzymes and chemotactic factors.

These metabolic events may be the initial stimulus for exercise induced injury, with loss of calcium homeostasis being a key perpetrator in the damaging process, setting off a chain of events. Depletion of cellular thiols and increased intracellular calcium may potentiate free radical generation, membrane lipid peroxidation and leakage of intracellular enzymes (Meydani and Evans, 1993).

Thus, evidence exists to implicate cytotoxic reactive oxygen species as an underlying etiology in exercise-induced muscle fatigue and bioenergetic enzyme down-regulation (Powers *et al.*, 1994). In order to deal with the potentially deleterious effect of reactive oxygen species, cells have evolved an elaborate system of defense mechanisms to prevent oxidative injury. These protective mechanisms include the enzymes SOD, catalase, and glutathione peroxidase. Presently, there is insufficient data available to describe how the human body

tolerates the accelerated production of free radicals and how the consequences of that chemistry may relate to the overall well being of exercising individuals (Jenkins, 1988). It is likely that increased cellular activity of one or more of these antioxidant enzymes will decrease the risk of cellular injury from free radicals.

Cytosolic Oxidative Stress

Although most people have concentrated on the role of the mitochondrial enzyme, Mn-SOD, in dealing with the oxidative stress associated with exercise, the role of the cytosolic enzyme, Cu,Zn-SOD, also deserves attention as the cytosol is also vulnerable to oxidative insults during exercise. There are three main sources of oxygen free radicals which pose cytosolic oxidative stress during exercise. These free radicals may come from (1) the mitochondria, where the oxygen free radicals may escape the mitochondrial scavenging enzymes and leak into the cytosol, (2) capillary endothelium, where a hypoxia/reperfusion situation is created during exercise, and/or (3) the inflammatory cells, where superoxide production is elicited by mediators generated during exercise (Meydani and Evans, 1993).

Under normal conditions, molecular oxygen which contains two unpaired electrons is reduced with four electrons and four hydrogen ions to form water in the mitochondria, with cytochrome oxidase acting as the final electron acceptor. However, 2-5% of molecular oxygen is involved in a step-wise one-electron reduction pathway which leads to the production of superoxide. This highly reactive species can then undergo further reactions to form the hydroxyl radical, hydrogen peroxide and finally water. These are normal by-products of cellular metabolism and their highly reactive nature threatens the integrity of other cellular components. The effects of these reactions over a lifetime have been postulated as contributing to the aging process.

During intense exercise, loss of cytochrome oxidase activity leads to a situation in which

the concentration of molecular oxygen exceeds the maximal reduction capacity of mitochondrial oxidative phosphorylation. This oxygen must therefore be reduced by an alternate pathway, such as reduction by semiquinones present in the mitochondrial membrane. This reaction generates superoxide (Meydani and Evans, 1993).

Reactive oxygen species are also produced during ischemia/reperfusion, a condition characteristic of the re-routing of blood during exercise from the sphlancnic circulation to the metabolically active tissues. Those tissues that are deprived of oxygen during exercise or physical exertion will be reoxygenated sometime after the cessation of intense activity. The mechanism of oxygen free radical generation during ischemia/reperfusion centres around the depletion of ATP in the hypoxic tissues. Degradation of ATP leads to xanthine production. During reoxygenation, xanthine dehydrogenase is converted to xanthine oxidase via mild proteolysis. This enzyme uses xanthine as a substrate to produce superoxide and uric acid (Meydani and Evans, 1993).

Exercise, which increases total body oxygen flux, can be potentially harmful to the body as virtually every biomolecule is susceptible to free radical damage. It is therefore intuitive that the body must adapt in some way to deal with this phenomenon. The cytosol of cells is not immune to free radical damage and thus must also protect itself.

Response of the Antioxidant System to Exercise

The role of antioxidants in combatting free radicals generated during exercise has been investigated and mixed results have been obtained. Evidence to support the claim that increased cellular activity of one or more antioxidant enzymes occurs during exercise, to prevent cellular injury from free radicals, has been actively sought. Some research indicates that in untrained animals antioxidant enzyme activity is greater in muscles with high oxidative capacity (Powers *et al.*, 1994). These observations have led investigators to postulate that

endurance training will show parallel increases in both oxidative and antioxidant enzymes. The relationship between physical activity and antioxidant levels is complicated by the aging process. It is not clear from the literature whether the two complement each other or act in opposite directions. The primary antioxidant enzymes, SOD, glutathione peroxidase, and catalase have each been reported to increase, decrease or stay the same with age. Thus, even the issue of the relationship between antioxidant defense mechanisms and age has not yet been resolved. The lack of consensus among researchers may be due to confounding variables affecting the results of the studies. For example, antioxidant capacity may also be a function of physical activity and therefore this factor must be considered when evaluating changes in antioxidant levels within a given population. Perhaps differences in subject characteristics such as their physical activity levels, gender, diet, etc. may account for the discrepant results being reported in the literature.

Training, Antioxidants, and Aging

Aging is a progressive accumulation of changes within tissues which increases the body's susceptibility to diseases, toxic effects of xenobiotics and death (Harman, 1981; Stohs, 1984). Aging has been attributed to DNA disturbances, a progressive breakdown of macromolecules, autoimmune disorders and damage incurred by endogenously generated free radicals (Harman, 1981; Farooqui *et al.*, 1987). As already mentioned, the Free Radical Theory of Aging postulates that the single basic cause of aging, modified by genetic and environmental factors is damage induced by free radical reactions. Antioxidants and antioxidant enzymes are the body's protection against reactive oxygen free radicals. It has been proposed that the rate of aging may be accelerated due to a decline in the antioxidant capacity of tissues. This would leave the body more vulnerable to oxidative damage.

One conspicuous result of free radical-mediated damage is lipid peroxidation of

polyunsaturated fatty acids. The consequences of this occurring include alterations of enzyme function (De Groot *et al.*, 1985), changes in membrane fluidity (Chia *et al.*, 1983) and morphological changes (De Groot *et al.*, 1985). The ease with which products of lipid peroxidation can be measured has made this technique almost the most exclusive focus of exercise studies (Halliwell and Grootveld, 1987). Consequently, researchers have examined thiobarbituric acid reactive substances (TBARS), hydrocarbon gases in expired air and conjugated dienes in organisms under exercising conditions with mixed results.

Although free radical chemistry has been analyzed from the perspective of its role in the aging process, it has not been until fairly recently that free radical chemistry has interested those involved in sports medicine or exercise physiology. Even less attention has been devoted to the study of the relationship of physical training, antioxidative capacity and aging.

However, since oxygen-centered radicals are produced in intermediate metabolism, it is not surprising that exercise accelerates their production (Jenkins, 1988). Thus, it is conceivable that the consumption of large quantities of ambient oxygen during exercise, which has been estimated to increase total body oxygen uptake by more than ten fold (Dillard *et al.*, 1978; Brady *et al.*, 1979; Davies *et al.*, 1982), can be potentially deleterious to the body (Jenkins, 1988). This is because the fine balance which exists in aerobic organisms between free radical production and protective mechanisms against such species may be perturbed by exercise. In fact, Davies *et al.*, (1982) using paramagnetic resonance techniques demonstrated that fatiguing exercise resulted in an increased radical signal in rat muscle and liver. It is important to note that this study was conducted on muscles taken from untrained animals.

The current literature indicates that the amount of damage is dependent on exercise intensity, training state of the individual and the tissue examined (Witt *et al.*, 1992). Hence, although physical exercise is known to be beneficial in many respects, free radical production

during exercise may stimulate oxidative damage which is evident in the muscles, liver, blood and perhaps other tissues of the body (Witt *et al.*, 1992).

Research into free radical chemistry and exercise suggests that there are adequate free radical scavenging enzymes available in tissues in the resting state of normal (young) sedentary individuals; however, intense physical activity in untrained individuals increases free radical production beyond the antioxidant capacity of the tissues, thereby leading to oxidative stress (Ji *et al.*, 1988). On the other hand, the physically active or exercise-trained individual is protected against such oxidative stress due to a substantial induction of antioxidant capacity which is acquired as an adaptive response to exercise (Ji *et al.*, 1988).

There are at least two mechanisms via which reactive oxygen species (ROS) may be produced during exercise (Witt *et al.*, 1992). One source is electron leakage (leading to $O_2^{\cdot-}$ production), probably at the ubiquinone-cytochrome b level of the electron transport chain (Boveris and Chance, 1973; Boveris and Cadenas, 1975; and Cadenas *et al.*, 1977). For example, Fridovich (1979) reported that $O_2^{\cdot-}$ is produced at the rate of 0.5 nmol/min/mg protein by mitochondrial membranes, 6.5 nmol/min/mg protein from ubiquinol cytochrome c reductase and 9.8 nmol/min/mg protein from NADH-coenzyme Q reductase. These data were derived from *in vitro* studies. Although no data is available for active tissue it is assumed that the rate of radical production is a function of oxygen flow through the tissues and therefore would be elevated under exercising conditions (Jenkins, 1988). This becomes particularly evident when one considers that whole-body oxygen consumption during exercise can increase 10-20 fold (Astrand and Rodahl, 1986) and the oxygen flux in individual muscle fibers may increase as much as 100-200 fold (Keul *et al.*, 1972). Thus, it is very likely that mitochondrial $O_2^{\cdot-}$ production during exercise is greatly enhanced.

The second source of oxygen free radicals during exercise is via a mechanism of ischemia-

reperfusion. Under exercise conditions, blood flow is shunted away from many organs and tissues (e.g., kidneys and splanchnic region) to be directed instead toward working muscles. Thus, part or all of these regions which are bypassed may experience hypoxia. Furthermore, during exercise at or above maximal oxygen uptake (VO_{2max}) and perhaps even at lower intensities, it is known that fibers within the working muscles also experience hypoxia (Witt, 1992). Cessation of exercise is accompanied by reoxygenation of these regions which in turn leads to the well known burst of ROS production that occurs during ischemia-reperfusion (Kellog III and Fridovich, 1975; Wolbarsht and Fridovich, 1989). Electron spin resonance techniques have directly demonstrated exercise-induced free radical production in skeletal muscles and liver of animals (Davies *et al.*, 1982; Jackson *et al.*, 1985).

Although the potential importance of free radical events for exercise physiology became evident in 1974 when McCord showed that oxygen-derived free radicals (i.e. $O_2^{\cdot-}$) were probably responsible for the inflammatory destruction of synovial fluid; interest in exercise and free radicals did not gain impetus until less than ten years ago when McCord (1985b) advanced the reoxidation theory of tissue damage (Kretzschmar and Muller, 1993). Since then studies have concentrated on elucidating the relationship between training and acute exercise on free radical chemistry.

Because exercise involves a chronic intermittent increased exposure to oxygen, it is of interest to discover whether the antioxidant system of trained individuals is capable of adapting to elevated oxidative stress.

Allesio and Goldfarb (1988) performed a study in rats to determine whether endurance training influenced the production of lipid peroxide (LPO) byproducts at rest and following an acute exercise run. The scavenger enzymes, CAT and SOD, were also examined to detect whether changes in lipid peroxidation were the result of alterations in enzyme activity both

at rest and after exercise. The training program (treadmill running) led to a 70% increase in oxidative capacity of rat leg muscles. After exercise the sedentary group displayed elevated LPO concentrations in the liver and white skeletal muscle fibre. This was not seen in the endurance trained group. CAT activity was found to be higher in the trained rats and SOD activity appeared to be unaffected either by acute or chronic exercise. These data suggest that training may lead to a reduction of LPO production during moderate intensity exercise perhaps due to the activation of antioxidant enzymes such as CAT.

Ji *et al.* (1988) investigated the effect of selenium deficiency, chronic exercise training and acute exercise on hepatic and skeletal muscle antioxidant enzymes (SOD, CAT, selenium dependent glutathione peroxidase (GPX) and glutathione-S-transferase (GST)) and tissue lipid peroxidation in rats. They found that antioxidant enzymes in liver and skeletal muscle are able to adapt to selenium deficiency and exercise in order to minimize free radical induced oxidative damage.

Research teams in Finland (Jen *et al.*, 1992 cited in Kretzschmar and Muller, 1993) and Germany (Marin *et al.*, 1993 cited in Kretzschmar and Muller, 1993) carried out the first long term study (1990-1991) on putative training effects on glutathione homeostasis. The study aimed to discover the influence of long term physical training on glutathione synthesizing capacity and glutathione concentrations in different organs (liver, muscle, lung) as well as the resulting plasma glutathione concentration. Hence, female beagle dogs were trained over a one year period to run on a 15 degree uphill inclined treadmill. A significant increase in the lung and gastrocnemius muscle glutathione concentrations was observed in trained animals. Also, a dramatic increase in glutathione synthesis was observed in the muscles of trained animals. Interestingly, this effect was found to be restricted exclusively to those muscles affected by the training regime. No training effect was observed in the liver

or lungs. The glutathione plasma levels were also higher in the trained dogs as compared to the untrained dogs despite no change in liver glutathione levels or hepatic glutathione synthesizing activity. The results of this study indicate that the glutathione system of skeletal muscle adapts very well to exercise training.

Kretzschmar and Muller (1993) studying the influence of training and exercise on plasma glutathione and lipid peroxides in humans also observed similar results. A major finding in their study was that the age-dependent decrease in plasma glutathione levels could be partly compensated for by training. They speculated that this effect may the result of increased glutathione export from skeletal muscle into circulation in the trained subject. This elevated plasma glutathione concentration may be of significance to other organs (i.e., lungs).

Furthermore, Quintanilha (1984) demonstrated that endurance-trained rats exhibited less erythrocyte hemolysis, which may indicate a lowered rate of lipid peroxidation. He also found an increase in antioxidative enzymes (i.e., GPX and SOD).

Data on the effect of an acute bout of exercise in untrained animals almost uniformally indicates a transient increase in lipid peroxidation (Jenkins, 1988). Brady *et al.* (1978) found that plasma TBARS levels rose significantly in horses after only a ten minute run. Studies conducted on rats have also indicated an increase in lipid peroxidation following unaccustomed exercise (Davies *et al.*, 1982; Jenkins *et al.*, 1983).

Gutteridge *et al.* (1985) have detected both copper and iron in sweat obtained from athletes immediately following exercise. These metals are capable of stimulating lipid peroxidation. This observation is noteworthy since Wibur *et al.* (1949) also found TBARS in human sweat. Gutteridge proposes that the body excretes malondialdehyde and metals through sweat as a mechanism employed to reduce the risk of lipid peroxidation.

Although the data concerning the relationship between CAT activity levels and exercise

has been somewhat controversial, the data regarding the effect of training on SOD is bit more consistent. Several researchers have reported an increase in SOD activity with training (Quintanilha, 1984; Jenkins *et al.*, 1984; Kanter *et al.*, 1985). Karlsson (1987) found an increase in skeletal muscle SOD in trained subjects. Higuchi *et al.* (1985) reported no increase in cytoplasmic SOD, but a 37% increase in mitochondrial SOD in fast and slow twitch red muscle and a 14% increase in this enzyme in white muscle.

Jenkins (1983) studied the influence of depressed CAT and SOD on muscular fatigue in experimental animals (frogs and rats). Animals were either injected with 3-amino-1,2,4-triazole (a CAT inhibitor) or diethyldithiocarbamate (a SOD inhibitor). These inhibitors did not effect an electrically stimulated contraction in frogs, but both compounds significantly reduced the time to fatigue in the rat soleus muscle.

Jenkins *et al.* (1984) also measured CAT and SOD in biopsies of human vastus lateralis muscle and reported significant correlations between both enzymes and VO_{2max}. This study appears to be the only published work to date on these enzymes in human tissue.

Although recently there has been a surge in interest in exercise-induced free radical chemistry, our knowledge in this area is still in its infancy. Thus far, it appears that acute (especially unaccustomed) exercise leads to an increase in the production of free radicals and subsequently an increase in lipid peroxidation. Endurance training may reduce lipid peroxidation in some tissues; however, no single mechanism can be advanced to explain this phenomenon as only relatively modest changes have been observed in the two primary antioxidative enzymes, SOD and CAT. Nonetheless, the accumulated data so far seems optimistic with respect to attenuating some of the antioxidant declines observed in aging individuals. Very limited data has been collected to date regarding the effect of aging on skeletal muscle antioxidant enzymes and its relationship to physical activity. However, the

research that has been conducted in this area seems promising. Ji *et al.* (1991) were the first to study the response of antioxidant enzymes to training in senescent animals. No significant changes in SOD or CAT activities following 10 weeks of endurance training were observed in senescent rats (mean age=27.5 months). However, it was noted that there was a tendency for the activity of both enzymes to be slightly elevated. GPX activity, on the other hand, increased dramatically (62%) in the deep portion of the vastus lateralis muscle (DVL) in old rats following endurance training. GPX induction was comparable to that observed in young trained rats. It was also observed that the decrease in muscle enzyme activity in senescent rats and the corresponding increase with training were associated with parallel changes in muscle protein content. The study was instrumental in showing that even senescent animals can elevate antioxidant enzyme levels through physical training.

In a 1992 study, Ji *et al.* reported that exhaustive exercise increased rat muscle glutathione disulfide content by 75% in hydroperoxide injected rats and 60% in saline injected rats (controls). Concentrations of the glutathione related amino acids glutamate, cysteine and aspartate were also found to be significantly elevated in the same muscle following exhaustive exercise. Strikingly, SOD, CAT, GPX, and glutathione reductase activities were also significantly elevated in muscle after exhaustive exercise with or without hydroperoxide injection. This study by Ji *et al.* is apparently the first report to demonstrate a uniform elevation of all antioxidant enzyme activities in response to exercise.

Experimental Aim and Design

Purpose

The objective of this study was to investigate the relationship between SOD and aging as a function of physical activity levels of subjects.

Hypothesis

It was hypothesized that all parameters of SOD investigated in the active elderly groups would be closer to the values obtained for the younger subjects. However, the values determined for the elderly inactive individuals were expected to show greater deviation from the mean values found in the younger subjects (i.e., a decrease in SOD activity).

Thus, it was believed that maintaining high physical activity levels would attenuate some of the physiological declines/changes which accompany the aging process. Also, individuals who exercise regularly, thereby subjecting their bodies to increased oxidative stress, were expected to show an adaptive response in which they displayed elevated antioxidant enzyme activity levels. It was predicted that the differences between active and inactive individuals would be more pronounced among the elderly.

It was expected that with aging a decrease in SOD activity would be observed. This decrease may either be the result of a decrease in SOD catalytic activity due to the synthesis of less efficient SOD molecules with age or, alternatively, it may be due to depressed SOD concentrations with age.

It was hoped that a measurement of SOD activity levels via the xanthine/xanthine oxidase/cytochrome c method and the energy of activation of the enzyme would resolve this uncertainty, as well as clarify some of the controversy surrounding changes in SOD with respect to aging.

Methods

SUBJECTS

129 healthy subjects (80 males and 49 females) ranging in age from 7-87 years were recruited for this study. These subjects were all community dwelling individuals who were neither smokers nor taking any regular, prescribed medications (which may interfere with the SOD assay). Each subject filled out a self-administered questionnaire which gathered information regarding typical weekly activities, such as amount of time spent per day walking, climbing stairs, playing sports and engaging in physical activity. This questionnaire was a modification of that of Paffenbarger *et al.*, 1983 (Appendix I).

SUBJECT GROUPS

Subjects were separated into groups based on physical activity levels (active/inactive), gender, and 20 year age brackets. The subjects classified into 20 year age groupings were further categorized as active or inactive:

GROUP 1: active 0-20 year olds

GROUP 2: inactive 0-20 year olds

GROUP 3: active 20-40 year olds

GROUP 4: inactive 20-40 year olds

GROUP 5: active 40-60 year olds

GROUP 6: inactive 40-60 year olds

GROUP 7: active 60-80 year olds

GROUP 8: inactive 60-80 year olds

GROUP 9: active 80-100 year olds

GROUP 10: inactive 80-100 year olds

Statistical analysis indicated that each of the activity groups were age-matched, as no statistically significant difference in mean ages was detected between the active and inactive groups within a given age bracket. Table 1 is a summary of the mean ages and physical activity levels of each of the above 10 groups also separated for gender.

BLOOD SAMPLES

7.5 mL whole blood samples were collected in heparinized tubes. These were obtained via venipuncture to the antecubital vein. These samples were used to determine whole blood Hb content, SOD activity and total protein concentration in the chloroform/ethanol extract.

RATIONAL FOR STUDYING ERYTHROCYTE SOD

Erythrocytes afford a useful model for investigating age-related alterations in antioxidant enzymes, particularly Cu,Zn-SOD which is abundant in these cells. The attractiveness of using red blood cells to study such changes is based on many factors: the red blood cell is exposed to high levels of oxidative stress, as its main function is the transport of oxygen; red blood cells are not contaminated by the mitochondrial isoenzyme, Mn-SOD (Marklund, 1974); red blood cells do not manufacture any new proteins (Glass and Gershon, 1981); red blood cells contain relatively high concentrations of Cu,Zn-SOD (Winterbourn *et al.*, 1975; Concetti *et al.*, 1976; Terada *et al.*, 1990); and degree of oxidative stress is readily determined in red blood cells by measuring malondialdehyde content (Joswiak and Jasnowska, 1985).

In erythrocytes, SOD protects against the oxidation of oxyhemoglobin to methemoglobin (Concetti *et al.*, 1976; Glass and Gershon, 1981), superoxide induced hemolysis (Concetti *et al.*, 1976; Glass and Gershon, 1981), and the peroxidation of membrane lipids (Glass and Gershon, 1981; Jozwiak and Jasnowska, 1985).

Erythrocyte membranes are particularly vulnerable to oxidative damage as they are characterized by a high content of polyunsaturated fatty acids. Studies have shown that the

extent of lipid peroxidation of red blood cell membranes is dependent on the age of the donor and that the rate of lipid peroxidation accelerates with increasing age (Joswiak and Jasnowska, 1985).

Lipid peroxidation has many irreversible, detrimental effects on the cell. For example, accumulation of malondialdehyde, one of the main by-products of lipid peroxidation, has been associated with changes in the activity of adenylate cyclase and protein kinase (Pfeffer and Sislocki, 1976), disturbances in aminophospholipid organization in the membrane bilayer (Jain, 1984), and alterations in the stability of hemoglobin molecules (Kikugawa *et al.*, 1984). Thus, the red blood cell seems to be a prime target of free radical attack as well as damage induced by decomposition products of oxygen free radicals.

Furthermore, the red blood cell is a good candidate for studying the relationship between aging and SOD because no detectable protein turnover occurs during the lifespan of a erythrocyte which is 110-120 days in young adult humans (Glass and Gershon, 1981; it should be noted, however, that the mean lifespan of erythrocytes in the circulation of old animals seems to be much shorter). Thus, the erythrocyte system offers a good model for investigating age-related changes in proteins. A loss of activity of many red blood cell enzymes with advancing cell age has been well-documented (see Beutler, 1975 for a review).

Finally, another appealing feature of using red blood cells in this study is the high concentration of Cu,Zn-SOD in erythrocytes. Winterbourn *et al.* (1975) have reported the presence of approximately 0.5 milligrams of SOD per gram of hemoglobin. However, this value may be too high as it has been observed that the nitroblue tetrazolium method in the presence of hemoglobin overestimates SOD activity (Concetti *et al.*, 1976). Marklund (1974) reported that a hemolysate of human blood contains 105 milligrams of Cu,Zn-SOD per litre of packed erythrocytes. This value agrees well with data obtained immunochemically.

Concetti *et al.* have also determined SOD content in normal red blood cells. Based on a study of 19 normal adults they found the mean SOD content to be 6.2 $\pm$ 1.4x10^{-15} g/RBC. This corresponds to an SOD concentration in whole blood of ~1 uM and a Hb/SOD weight ratio in blood of 2-5x10^{3}. A further calculation reveals that there is approximately one molecule of SOD per 2 500 molecules of hemoglobin tetramers. Terada *et al.* (1990) have reported that the Cu,Zn-SOD concentration in human blood cells to be 1.43$\pm$0.11 ng/10^{5} cells based on five observations of samples measured in duplicate. This value is in relatively close agreement with that reported by Concetti *et al.* (1975).

PARAMETERS DETERMINED

Erythrocyte Cu,Zn-SOD total activity, extract protein concentration (via the Lowry and Bradford methods) and whole blood hemoglobin concentrations were determined for all subjects. Also, SOD activity was determined at two different temperatures in order to elucidate whether physical properties of the SOD enzyme differed between the groups.

PREPARATION OF SUPEROXIDE DISMUTASE EXTRACT

1.) 2 mL of heparinized blood is centrifuged at 3000 rpm (1086 g) for 10 minutes at 0-4°C in an SS-34 Sorval centrifuge.

2.) Plasma is carefully separated and the top buffy coat is removed and discarded.

3.) 0.5 mL of erythrocytes is carefully removed from the bottom of the centrifuge tube and lysed with 4.5 mL of ice cold water.

4.) Hemoglobin and Mn-SOD in the plasma and serum is removed by adding 3 mL of chloroform and 5 mL of ethanol according to the Tsuchihashi method. This step is critical as Hb interferes with the SOD assay by counteracting the inhibitory effect of SOD on cytochrome c reduction. This step is repeated if solutions are not clear following step 6.

5.) The contents of the centrifuge tube are vigorously vortex-mixed for at least 1 minute and a thick white precipitate accumulates.

6.) The mixture is centrifuged at 12 000 rpm (~18 000 g) for 60 minutes at 0-4 C.

7.) The almost clear supernatant is diluted by a factor of 100 (This large dilution precludes the necessity for dialysis to remove low molecular weight SOD-like substances).

8.) 0-480 uL of the diluted solution is used to assay for Cu,Zn-SOD activity.

*Note: the recovery of a standard of pure Cu,Zn-SOD added to the hemolysate is ractically complete.

ASSAY OF SUPEROXIDE DISMUTASE

The xanthine/xanthine oxidase/cytochrome c indirect assay of SOD follows the procedure outlined by L'Abbe and Fisher (1990) with minor modifications.

Reagents:

-sodium carbonate buffer

-ferricytochrome c (type VI from horse heart, SIGMA; MW = 12 384 g/mol)

-xanthine (2,6-dihydroxypurine, 99-100%, SIGMA; MW = 152.1 g/mol)

-xanthine oxidase (xanthine: oxygen oxidoreductase, Grade I from buttermilk, SIGMA; 28 mg protein/mL, 0.68 units/mg protein)

-Cu,Zn SOD (SIGMA)

Stock solutions:

-20 mM sodium carbonate buffer, pH 10.0 containing 0.1 mM EDTA (The assay for Cu,Zn-SOD is 17X more sensitive at pH 10.0 than at pH 7.8 and Mn-SOD is inhibited at this pH).

-500 uM ferricytochrome c or 0.16 g/25 mL (note: this is light sensitive and must

be stored refrigerated in the dark by covering with foil).

-1.0 mM xanthine

Reagent Mixture:

-93 mL sodium carbonate buffer

-2 mL cyt c solution

-5 mL xanthine solution

*Note: this mixture is stable for several weeks when kept in the refrigerator. Since it contains cyt c, it must be covered with foil to prevent light penetration.

SOD Activity Assay:

Superoxide is generated by the following reaction:

$$\text{xanthine} + 2\ O_2 + H_2O \text{ ---> uric acid} + 2\ O_2^{\cdot-} + 2\ H^+$$

This reaction is monitored at 415 nm on a Hewlett Packard spectrophotometer as an increase in absorbance due to the reduction of cyt c.

SOD which catalyzes the dismutation of superoxide ($2O_2^{\cdot-}$ ---> $H_2O_2 + O_2$), inhibits the above reaction.

% Inhibition:

% inhibition = (control rate - sample rate)/control rate x 100%,

where control rate equals the rate in the absence of SOD and sample rate equals the rate in the presence of SOD.

Unit SOD Activity:

Activity calculation: units SOD/mL packed RBC = 1000uL/(uL/unit) * 1000, where the first term represents a correction for the dilution in the cuvette and the second term is a correction for the dilution of the extract to the RBC.

One unit of SOD activity is defined as the amount of enzyme which inhibits the rate of cyt c reduction by 50% under the conditions specified for a particular system in a 1 mL reaction volume.

The specific activities determined via the assay used in this study will be ~ 17 fold greater than that determined by McCord and Fridovich (1969). This increase is due to the use of a higher pH and a more sensitive wavelength.

KINETIC ANALYSIS

For each subject SOD activity levels were determined via a continuous multiple point assay. The volume of chloroform/ethanol extract containing SOD was varied, keeping all other conditions of the assay constant. SOD was assayed via an inhibition reaction in which the presence of SOD competitively inhibits the reduction of cytochrome c by the superoxide radical. A graph of %Inhibition vs uL of Cu,Zn-SOD (i.e. Ch/EtOH extract) yields a hyperbolic curve. The equation of this curve is the following:

$$\%\text{Inhibition} = 100[\text{SOD}]/(\text{Km} + [\text{SOD}]), \text{ where } 100 = \%\text{Inhibition maximum}$$

The above equation is analogous to the Lineweaver-Burke equation:

$$v = Vm\ [S]/(Km + [S])$$

where v = velocity, [S] = substrate concentration (i.e., SOD concentration), Vm = velocity maximum (in the SOD assay this is ~ 100% at high SOD concentrations), and Km = [S] at which the enzyme is working at 1/2 Vm (which in the SOD assay equals the concentration of SOD which gives 50% inhibition of the xanthine oxidase reaction and is equivalent to one unit of SOD activity).

The data was plotted as 1/% Inhibition vs 1/uL of Cu,Zn-SOD. The equation representing this graph is the following:

$$1/\%\ \text{Inhibition} = \text{Km}/100 * 1/[\text{SOD}] + 1/100$$

This is analogous to the double reciprocal Lineweaver-Burke plot:

$$1/v = Km/Vm * 1/[S] + 1/Vm, \text{ where } Vm = kcat\ Et$$

Thus, the slope of this line equals Km/(kcat Et). The term kcat/Km represents the catalytic efficiency of the enzyme and Et represents the total amount of the enzyme, SOD. Hence 1/slope = (kcat Et/Km)', a kinetic parameter which in this case is an indicator of SOD activity:

1/slope (of 1/%Inhibition vs 1/uL extract) = (kcat Et/Km)' = relative SOD activity

ARRHENIUS PLOT

The Arrhenius equation:

$$\ln kcat = -Ea/(RT) + \ln A = -Ea/R * 1/T + \ln A,$$

where R = the universal gas constant and Ea represents the energy of activation. The above equation is analogous to the following equation:

$$\ln \%Inhibition = -Ea' * 1/T + \ln A,$$

where %Inhibition is a function of kcat (the rate constant).

The competition for the superoxide radical by cytochrome c and Cu,Zn-SOD occurs at a higher rate at higher temperatures. Thus, the assay in effect, is measuring the relative ability of an individual's Cu,Zn-SOD to compete with cytochrome c. Since the same cytochrome c was used in all assays, any factors influencing this competition will be attributable solely to the SOD enzyme. However, because this method is a modification of the classical Arrhenius plot, Ea cannot be calculated in kcal/mol. Nonetheless, the assay qualitatively reflects any changes in Ea or kcat/Km which will result from any modification of the physical properties of the Cu,Zn-SOD enzyme.

PROTEIN DETERMINATION

Total protein in SOD extracts was determined by two different methods: (1) the Lowry method (Lowry *et al.*, 1951) and (2) the Bradford microprotein assay (Bradford, 1976).

Hemoglobin concentration was determined via the Drabkin method (Drabkin and Austin, 1935).

SDS GEL ELECTROPHORESIS

The purity of the chloroform/ethanol extract was analyzed via polyacrylamide gel electrophoresis. SDS slap mini-gels were run in 5% acrylamide. Soon after development, the pattern of bands on the gel were sketched. Two lanes contained the standards SOD (16 kDa), serum albumin (67 kDa) and glycogen phosphorylase (100 kDa). The distance of migration of the known bands were plotted vs log molecular weight. From this curve, molecular weight values for the unknown proteins were estimated and tentative assignments made.

STATISTICAL ANALYSIS

The SAS statistical program was used to perform an ANOVA (analysis of variance) in order to determine whether there were significant differences ($p<0.05$) in mean values for (kcat Et/Km)', hemoglobin concentration, physical activity levels, extract Lowry protein concentration, extract Bradford protein concentration and Arrhenius slope between any of the subject groups separated according to age, physical activity levels or gender. Statistically significant differences at the $p<0.05$ level were examined using the t test (LSD). The output of this analysis is presented in Appendix J. Simple statistics were also performed on all experimental parameters and Pearson correlation coefficients were determined. The output of this correlation analysis is presented in Appendix K. In addition, a regression analysis was performed to derive an equation which related (kcat Et/Km)' as a function of other influencing factors (Appendix K).

Means and standard deviations were calculated for all experimental parameters. The SAS statistical program was used to calculate the standard deviations of the slopes and y-intercepts of each double reciprocal plot of 1/%Inhibition vs 1/uL of Cu,Zn-SOD. This program was also used to force a best fit line through a 0.01 y-intercept (since %Inhibition maximum = 100% at high SOD concentrations, it can be assumed that 1/Vm = 0.01). From this a value for corrected slope was obtained. This showed strong agreement with the values obtained from the original double reciprocal plots when the y-intercept was not forced. Thus, in all calculations, comparisons, and plots the raw value for slope was used.

RESULTS

Lowry vs Bradford Method of Protein Determination

Although the Lowry method of protein determination is perhaps the most widely used as an index of total protein, the protein concentrations in the Ch/EtOH extracts of subject's blood was also measured via the Bradford microprotein assay. The Bradford method allowed an additional check on the values obtained for protein content in the extracts. It was also useful in differentiating between total extracted protein and other reducing agents (e.g., sulfhydryl and disulfide containing compounds) which are picked up by the Lowry method (Carmen and Lagunas, 1970). Figure 1 is a plot correlating protein concentration values in the extract obtained by the Bradford and Lowry methods of protein determination. From this graph it is evident that there is strong agreement between the values obtained for total extract protein concentration between the two methods (r=0.96). However, the values obtained by the Lowry method were approximately 16% higher than values obtained by the Bradford method. This discrepancy can be attributed to other reducing agents besides proteins (e.g., cysteine and glutathione) being detected by the Lowry method, but not the Bradford method of protein determination.

Determination of (kcat Et/Km)' via a Multiple Point SOD Assay

Appendix L is a collection of the double reciprocal plots of all the subjects used in this study. The SAS statistical program was used to calculate the standard deviations of both the slope and intercept of these plots. Also, a best fit line through a 0.01 y-intercept (assuming %Inhibition maximum = 100%, since 100% inhibition is achieved at high SOD concentrations). From this, a value for corrected slope was obtained. This showed strong agreement with the values for slope obtained from the original double reciprocal plots when the y-intercept was not forced. Appendix M is a summary of slope, corrected slope, and y-intercept values with

their respective standard deviations for all subjects.

The relationship between (kcat Et/Km)' and the following parameters: extract Lowry protein concentration, extract Bradford protein concentration, whole blood hemoglobin concentration, age and physical activity was examined. Appendix N is a summary of all of the parameters determined for each subject. SOD activity per mL packed RBC was determined from the value of (kcat Et/Km)'. A plot of (kcat Et/Km)' vs units SOD/mL RBC (Figure 2) yielded a 100% correlation. This should be the case since (kcat Et/Km)' differs from SOD units/mL by only the dilution factor.

(kcat Et/Km)' vs Extract Protein Concentration

A plot of (kcat Et/Km)' vs extract protein concentration determined by the Lowry method yielded (Figure 3) a linear graph (r=0.88). Thus, SOD activity was found to be proportional to protein content of the Ch/EtOH extracts. This would be expected, since the relative efficiency of SOD purification should be proportional to the purification of those proteins which can survive the harsh Ch/EtOH extraction procedure. Therefore, these graphs indicate the relative purity of SOD in the Ch/EtOH extract. The slope of the plot of (kcat Et/Km)' vs extract protein concentration is determined by (1) the catalytic efficiency of SOD, (kcat/Km)', (2) the amount of SOD present, (Et), and (3) the amount of Lowry protein surviving the chloroform/ethanol extraction procedure. Total amount of SOD is influenced by rate of synthesis (gene expression) and rate of degradation. Amount of proteins surviving the extraction procedure depends on overall cellular protein synthesis rates and the integrity of the proteins. Other work has provided evidence that oxidative damage may reduce the concentration of Lowry protein surviving the extraction procedure (Sheila Manhas, personal communications). Changes in any of the above mentioned factors will affect the slope of (kcat Et/Km)' vs extract Lowry (or Bradford) protein concentration in a predictable way. An

increase in SOD activity or a decrease in extract protein concentration will both lead to an increase in the slope of this graph. Due to the divergent characteristics of the study population in terms of physical activity levels, gender and age which may affect the factors influencing the slope of this graph, (kcat Et/Km)' vs extract Lowry protein concentration was plotted separately for the different groups of subjects.

Influence of PHYSICAL ACTIVITY on the Slope of (kcat Et/Km)' vs Extract Protein Concentration

Noteworthy, is the finding that the slope of relative SOD activity, (kcat Et/Km)', plotted against protein concentration is higher for the active individuals (those expending > 1500 kcal/wk) than the inactive individuals (those expending less than 1500 kcal/wk). These slopes were 0.091 (r=0.091) for the active group (Figure 4) as compared to 0.082 (r=0.88) for the inactive group (Figure 5). Hence the active group displayed on average an 11% greater SOD specific activity. Table 2 indicates that the mean value of (kcat Et/Km)' is higher for the active subjects (0.64, n=51) compared to the inactive subjects (0.46, n=45) and this difference was found to be significant (p=0.0003). The mean value of extract Lowry protein concentration for the active group (6.57 ug/mL, n=60) is also higher than that of the inactive group (6.16 ug/mL, n=52), but the difference was not found to be statistically significant (Table 2).

Influence of AGE on the Slope of (kcat Et/Km)' vs Extract Protein Concentration

Plots were also graphed of (kcat Et/Km)' vs the various age categories to evaluate differences based on age alone (i.e., ignoring physical activity levels). The slope of (kcat Et/Km)' vs extract Lowry protein concentration for the 0-20 year old subjects (Figure 6) was found to be 0.068 (r=0.79). That for the 20-40 year old subjects (Figure 7) was 0.10 (r=0.90). The slope for the 40-60 year old subjects (Figure 8) was found to be 0.092 (r=0.90) and for

the 60-80 year old subjects (Figure 9) it was 0.083 (r=0.86). Due to insufficient subjects over the age of 80 for whom Lowry protein values had been measured, a similar plot for the 80-100 age category was not graphed. A monotonic trend with age was not observed because both SOD activity and total extract protein concentration go down with age and these factors have opposite effects on the slopes measured.

Influence of AGE and PHYSICAL ACTIVITY on the Slope of (kcat Et/Km)' vs Extract Protein Concentration

To examine the joint influence of both physical activity levels and age on the relationship between (kcat Et/Km)' and extract protein concentration, linear plots of (kcat Et/Km)' vs extract Lowry protein concentration were graphed in which subjects in each age category were further separated according to activity levels. The slope of this plot for the 0-20 year old active subjects (Figure 10) was found to be 0.073 (r=0.83). That for the inactive subjects in this age bracket (Figure 11) was 0.052 (r=0.82). The slope of the inactive group of 0-20 year olds may be inaccurate due to a low n value (4 subjects). For the 20-40 year old active group, a plot of (kcat Et/Km)' vs extract protein concentration (Figure 12) yielded a slope of 0.10 (r=0.94) and the corresponding slope for the 20-40 inactive group (Figure 13) was found to be 0.076 (r=0.87). This slope for the active 40-60 year olds (Figure 14) was 0.082 (r=0.75) and for the inactive 40-60 year olds (Figure 15) it was 0.097 (r=0.95). The slope of (kcat Et/Km)' vs extract Lowry protein concentration of the active 60-80 year olds was 0.077 (r=0.90) (Figure 16) and 0.073 (r=0.86) for the inactive 60-80 year olds (Figure 17). Similar plots for the active 80-100 year olds and the inactive 80-100 year olds were not graphed due to insufficient Lowry protein data for these groups and high variability within these groups.

The ratios of (kcat Et/Km)' to total Lowry protein show a complex effect as a function

of age in both the active and inactive groups. The active group has already been shown (Figure 4) to have a higher overall slope (0.91) compared to the inactive group (Figure 5) (0.082). This shows that in general active people have a higher ratio of SOD to other extracted proteins. However, the ratio of (kcat Et/Km)' to total Lowry protein peaks at middle age. The same middle age phenomenon is seen for the inactive group. The low ratio of (kcat Et/Km)' to Lowry protein for young people can be attributed to the presence of copious amounts of Lowry protein due to the absence of free radical damage (Figures 26 and 27). In older people the ratio of (kcat Et/Km)' to Lowry protein again becomes low because SOD activity is decreasing at a faster rate compared to other extract proteins in general (Figure 19).

Relationship between Hemoglobin Concentration and (kcat Et/Km)'

A plot of (kcat Et/Km)' vs whole blood hemoglobin concentration (Figure 18) showed no correlation between these two parameters (r=0.09). The mean value of hemoglobin concentration for the population studied was 15.4 g/dL (n=117) for all subjects.

Influence of AGE and PHYSICAL ACTIVITY on (kcat Et/Km)'

Bar graphs comparing the various parameters to age and physical activity revealed some interesting relationships. A bar graph of (kcat Et/Km)' vs age and physical activity (Figure 19) indicated a decrease in the former variable with respect to increasing age. However, within each age category the active individuals (physical activity index > 1500 kcal/wk) displayed higher levels of SOD activity compared to the relatively inactive individuals (physical activity index < 1500 kcal/wk). Figure 19 shows that the value of (kcat Et/Km)' is relatively constant up to approximately 40 years of age and then there is a steady drop in this parameter with advancing age. These results indicate that (kcat Et/Km)' is higher for the active people as compared to the inactive people and it is also higher for the young subjects as compared to the older ones.

(kcat Et/Km)' Active > (kcat Et/Km)' Inactive

(kcat Et/Km)' Young > (kcat Et/Km)' Old

(kcat Et/Km)' Active people under 40 years >>> (kcat Et/Km)' Active people over 40

There appears to be a dramatic decrease in SOD activity after the age of 40, especially among the active subpopulations. Figure 19 indicates a decline in (kcat Et/Km)' with age for both the active and inactive subgroups. Although, the value of (kcat Et/Km)' is always higher for the active group as compared to the inactive group, the declines in the parameter as a function of age seem to parallel each other. The greatest decrease in (kcat Et/Km)' occurs around age 40 and the difference in (kcat Et/Km)' between the groups separated for activity seems to be greatest in the younger individuals (<40 years) than in the older subjects (>40 years). The value for (kcat Et/Km)' is approximately 35-45% greater in the active young people compared to inactive young people; whereas, it is 15-35% higher in the old active people compared to old inactive people. The 40-60 year old active subjects had a lower mean (kcat Et/Km)' value than even the inactive younger individuals. Thus, physical activity could not overcome the advantages of youth, but it did attenuate some of the age-related declines in the parameter (kcat Et/Km)', which is analogous to SOD activity. However, in old age, the benefits of exercise appear greater as the 80-100 year old active individuals were found to have a higher (kcat Et/Km)' value than inactive 40-60 year olds. Therefore, activity was seen to bridge a 40 year age gap in this case. This observation agrees with the results of an ANOVA statistical analysis comparing (kcat Et/Km)' values between subject groups separated for age and physical activity (Appendix T). This analysis revealed that (kcat Et/Km)' differed significantly ($p<0.05$) between the younger active groups (active 0-20 and 20-40 year olds) and all other groups (except the inactive 0-20 year olds). Thus around 40 years, the value of (kcat Et/Km)' drops significantly, and even relatively high activity levels are not

sufficient to maintain the (kcat Et/Km)' values at the level of young active people.

Influence of GENDER on (kcat Et/Km)'

A bar graph of (kcat Et/Km)' vs age sorted for gender (Figure 20) indicates that (kcat Et/Km)' is generally higher in males than it is in females. The mean value for (kcat Et/Km)' for all male subjects is 0.57 (n=58) and for females it is 0.54 (n=38). This difference is not significant (Table 3). The sex specificity is more pronounced in the younger age categories (0-20 and 20-40 years) than in the older categories. In fact, there appears to be no significant difference between males and females in the 40-60 and 60-80 year old age categories. The mean value of (kcat Et/Km)' for the 80-100 Inactive age category is skewed due to a small n value.

When (kcat Et/Km)' vs age was only considered for the male population (Figure 21) it was clear that active males consistently across the age spectrum had higher levels of SOD activity than the relatively inactive males. The same was found to be generally true when only the female population was considered (Figure 22). Active females had considerably higher SOD activity than relatively inactive females. The mean value for (kcat Et/Km)' for the 80-100 year old inactive females is skewed due to insufficient subjects in this category.

When only the active population of males and females was considered (Figure 23), the bar graph of (kcat Et/Km)' vs age revealed that active males generally have higher SOD activity levels than active females up to approximately the 40 year old mark, after which the SOD levels of the active males and females become comparable.

A similar trend was observed when only the inactive population of males and females was considered. A bar graph of (kcat Et/Km)' vs age (Figure 24) indicated lower SOD activities among inactive females as compared to inactive males. From the mean values of each of the ten groups separated on the basis of 20 year age intervals and gender, it appears

that the males on average have a 20 year "advantage" over females in terms of SOD activity levels (with the exception of very old males, i.e. those over age 80). Again, due to a low n value for the 80-100 Inactive Females category, the mean value of (kcat Et/Km)' for this group appears to be skewed. Figure 25 is a summary of bar graphs 23 and 24. It allows a direct comparison of the mean (kcat Et/Km)' values of subjects separated for both age and physical activity. Although males of a comparable activity level consistently seem to have higher (kcat Et/Km)' values, this value in active females is greater than that of inactive males. Thus activity can successfully attenuate some of the gender differences.

Influence of AGE and PHYSICAL ACTIVITY on Extract Protein Concentration

A bar graph of Lowry extracted protein vs age (Figure 26) also revealed an interesting relationship. Protein concentration is seen to steadily decline with age. The biggest drop is seen in the very old age (greater than 80 years). The mean value of extract Lowry protein concentration of the 80-100 year old subjects was found to be 43% lower than that of the 0-20 year olds. When the subjects belonging to each age bracket were further separated for physical activity levels, the trend persisted. Figure 27 shows that protein concentration steadily declines with age in both active and inactive individuals. Appendix Q indicates that the mean values for extract Lowry protein concentration differ significantly ($p<0.05$) between the young (< 40 years) subjects and the old (> 40 years) subjects. Clearly extract Lowry protein concentration declines with age, but no significant difference exists between individuals of different physical activity levels within a given age bracket. However, active people as a whole seem to have slightly more extract Lowry protein concentration than do inactive people as is evidenced by a bar graph of Lowry protein concentration vs the active and inactive populations (Figure 28). The mean value for the active group (6.57 ug/mL, n=60) is approximately 7% higher than the mean value for the inactive group (6.16 ug/mL, n=52).

This difference was not found to be significant (Table 2). Thus, the drop out of Lowry protein seems to be more age dependent than dependent on physical activity levels since no significant difference was noted between the means of the active and inactive people in each age group (although the mean value of total extract Lowry protein in the active group as a whole was higher than that of the inactive group as a whole).

A bar graph of the mean (kcat Et/Km)' values vs active and inactive populations (Figure 29) yields a similar result as this value is higher for the active group (0.64, n=51) than the inactive group (0.46, n=45) by about 39%.

A bar graph of Lowry protein concentration in extracts of male and female subjects (Figure 30) show that protein concentration is higher in males (6.75 ug/mL, n=69) than in females (5.80 ug/mL, n=43) by 16%. This difference is not significant (Table 3). Similarly, a bar graph of the mean (kcat Et/Km)' values of males vs females (Figure 31) also indicates higher values for males (0.57, n=58) than females (0.54, n=38). Although the value for males is 7% higher than that of females, the difference is not significant (Table 3).

Relationship of Hemoglobin concentrations with AGE and PHYSICAL ACTIVITY

A bar graph of hemoglobin concentration vs age and physical activity (Figure 32) shows no correlation between these parameters. However, statistical analysis indicates that a weak positive relationship exists between hemoglobin concentration and age (p=0.026). Also, males were found to have a higher hemoglobin concentration than females (16.2 g/dL males, n=69 vs 14.2 g/dL in females, n=46). This difference was statistically significant (p=0.0001; Table 3). No significant difference was noted between the hemoglobin concentration of the active and inactive subjects (Table 2).

Arrhenius Slope

A temperature study was conducted in order to elucidate changes in physical properties of the SOD enzyme, by a determination of the catalytic efficiency of SOD (which is related to energy of activation) from the Arrhenius slope. However, no significant difference was noted in the values obtained for Arrhenius slope and any subcategory (i.e., no difference between the active (-470.2, n=49) and inactive subgroups (-395.8, n=31), Table 2; no difference between the male (-475.2, n=52) and female (-378.5, n=28) subgroups, Table 3; no difference between the 20 year spread age subgroups, Appendix J; and no difference between the groups separated for both age and physical activity, Appendix J). We can therefore conclude that all differences shown in SOD activity in this study are a result of increased protein concentration and not due to covalent allosteric alterations that could modify the catalytic efficiency of SOD.

SDS Gel Electrophoresis

Figure 33 is a drawing of the gel obtained for two subjects,one young and the other old. From the gel it became evident that the Ch/EtOH extracts were relatively clean and not contaminated by a whole slew of erythrocyte proteins. The tentative identification of proteins present in the extract (based on R_f values which correspond to protein molecular weights) is the following: The 14 kDa band was assigned as calmodulin, the 16.5 kDa band is probably a mixture of SOD and Hb (Hb is a 64 kDA tetramer and SOD is a 32 kDa dimer), the 35 kDa band (present only in the extract of the older subject) was assigned as glyceraldehyde-3 phosphate dehydrogenase (which may associate with the RBC membrane), the 54 kDa band was assigned to catalase and the 63 kDa band was assigned to glutathione reductase. The standards used for comparison were SOD (16.5 kDa), serum albumin (67 kDa), and glycogen phosphorylase (100 kDa). The gel was overstained for the older subject and this may be the

reason for the 35 kDa band (which was fairly weak) being detected only in the older subject.

Statistical Results

Tables 2-4 and appendices O-Y summarize the statistical analyses performed on data in this study. Table 2 lists the mean values of all experimental parameters comparing the active and inactive subjects. If a significant difference between the two groups exists within a 95% confidence interval, then a p value is indicated. Significant differences were found to exist between the active and inactive groups for the parameters (kcat Et/Km)' and physical activity levels.

Similarly, Table 3 is a summary of mean values for all experimental parameters comparing the male and female subject groups to each other. Again, p values were provided if a significant difference was found between the groups within a 95% confidence interval. This was found to be the case for hemoglobin concentrations and extract Bradford protein concentrations between the two groups.

Appendices O-S summarize differences in experimental parameters between groups separated into twenty year age brackets. Significant differences between groups is indicated with an asterix. Comparisons of significance are made at the p=0.05 level. Appendix O is a summary of the differences in (kcat Et/Km)' between the different age categories and it shows that (kcat Et/Km)' is significantly different ($p<0.052$) between the younger (< 40 years) groups and the older groups (> 40 years).

Appendix P is a summary of differences in physical activity levels between the age subcategories. It indicates that when subjects were classified as active or inactive based on a physical activity index cutoff of 1500 kcal/wk, there was no significant difference in physical activity levels between the groups (with the exception of the 0-20 year olds who differed significantly in their activity levels as compared to the 40-60 and 80-100 year olds).

This is desireable, since it indicates that the age subgroups are matched for physical activity. Thus, comparisons of experimental parameters between groups separated only for age will not be unduly influenced by gross differences in physical activity levels between the groups.

Appendix Q compares extract Lowry protein concentration between the various age groups. This table clearly depicts that extract Lowry protein concentration differs significantly between the younger subjects (< 40 years) as compared to the older subjects (> 40 years).

Appendix R summarizes differences in extract Bradford protein concentration between the various age groups. From this it is evident that extract Bradford protein concentration of the oldest subgroup (80-100 years) differs significantly form the rest of the population studied (i.e., all other age categories). Also, the extract Bradford protein concentration for the 0-20 year olds was significantly different from those of individuals greater than 40 years of age.

Appendix S summarizes differences in hemoglobin concentration between the various age groups. No significant difference was noted between the groups with the exception of the 60-80 year olds who differed with respect to hemoglobin concentration in comparison to individuals less than 40 years.

No significant difference was found in Arrhenius slope values between subject groups separated for age.

Appendices T-Y summarize differences in experimental parameters between subject groups separated for age and physical activity. Significant differences between groups within a 95% confidence interval are indicated with an asterix. Appendix T indicates differences in (kcat Et/Km)' between groups separated for age and activity. From this it can be seen that young active individuals (< 40 years) have significantly different (kcat Et/Km)' values than older age categories (> 60 years) regardless of physical activity levels.

Appendix U summarizes differences in physical activity levels between subjects separated

for age and activity. It shows that the physical activity levels between all the active groups is significantly different from all the inactive groups (except the active 80-100 year olds, which are not significantly different from the inactive groups).

Appendix V provides a comparison of extract Lowry protein concentration values between subject groups separated for age and physical activity. A significant difference was noted between the young subjects (< 20 years active and inactive) as well as the active 20-40 years olds when compared to old subjects (>80 years active and inactive) and the 60-80 year old inactive individuals. Thus, activity appears to be able to compensate for the 40 year age gap between the 20-40 and 60-80 year olds, as there was found to be no significant difference in extract Lowry protein concentration between the active 60-80 year olds and the inactive 20-40 year olds.

Appendix W is a similar summary as that in Appendix V, but in this case differences in extract Bradford protein concentrations are being compared. The results are identical to those for extract Lowry protein concentration differences between the various groups.

Appendix X summarizes differences in hemoglobin concentration between groups separated for age and physical activity. Although differences do exist between various groups, no consistent pattern is evident.

Appendix Y compares age differences between subject groups separated for age and physical activity. It clearly indicates that within a given age bracket, the ages of subjects classified as either active or inactive do not differ significantly. This is useful in showing that the physical activity subgroups of each age category are age-matched.

It is noteworthy that no significant difference existed in Arrhenius slope values between the subject groups separated for age and physical activity.

Finally Table 4 is a summary of the Pearson correlation matrix of the experimental

parameters (kcat Et/Km)', age, hemoglobin concentration, physical activity levels, extract Lowry protein concentration, extract Bradford protein concentration, and Arrhenius slope. Significant correlations were noted between (kcat Et/Km)' and the following parameters: age ($p=0.0001$), physical activity ($p=0.0001$), extract Lowry protein concentration ($p=0.0001$), and extract Bradford protein concentration ($p=0.0001$). Age was also found to be correlated with hemoglobin concentration ($p=0.03$). Physical activity was correlated with extract Lowry protein concentration ($p=0.03$) and extract Bradford protein concentration ($p=0.05$). Extract Lowry protein concentration was strongly correlated with extract Bradford protein concentration ($p=0.0001$).

When a multivariate regression analysis was performed to determine which parameters were predictors of (kcat Et/Km)', only age and physical activity levels appeared to be major influencing factors (Table 4). Thus, the linear equation derived from the data was the following:

(kcat Et/Km)' = -0.00188 * Age (years) + 0.000014 * Physical Activity (kcal/wk).

Hence, the decrease in (kcat Et/Km)' due to one additional year of life was found to be exactly compensated by a 134 kcal/wk increase in physical activity levels. Appendix Z which is a trend analysis, where age and physical activity are each plotted against SOD activity, supports the finding that age is a negative predictor of SOD activity and physical activity is a positive predictor of SOD activity. The graphs in Appendix Z show that with advancing age there is a curvilinear decline in SOD activity and with increasing levels of physical activity there is a curvilinear increase in SOD activity.

DISCUSSION

Relationship of SOD to age and physical activity

The objective of the present study was to elucidate the relationship between age, physical activity and the cytosolic antioxidant enzyme, Cu,Zn-SOD. A sample of 129 human volunteers, ranging in age from 7 to 87 and in which there was a representative sampling of both males and females, was used in the study. Thus, the influence of gender on the above parameters could also be investigated. Superoxide dismutase was partially purified from erythrocytes and physical activity levels of subjects was quantified from a self-administered questionnaire. Subjects were grouped in twenty year age intervals and further subcategorized as Active (expend > 1500 kcal/wk) or Inactive (expend < 1500 kcal/wk).

Both graphical and statistical analysis of the results indicated a significant relationship between the kinetic parameter (kcat Et/Km)' and both age and physical activity ($p = 0.0001$ in each case). Age was found to be a negative predictor of (kcat Et/Km)' (a parameter which is directly proportional to units of SOD activity) and physical activity, a positive predictor of (kcat Et/Km)'. Thus, younger individuals were found to have higher SOD activity than older individuals and active individuals had 39% higher SOD activity than inactive individuals overall. These results suggest that with age there is a loss of SOD activity, but this loss can be attenuated somewhat with exercise. These results agree with other reports in the literature regarding the response of antioxidants to exercise. Ji and Fu (1992) studying the response of several antioxidant enzymes to exercise found significantly elevated levels of glutathione peroxidase, glutathione reductase, SOD and catalase activities in rat muscle. None of the hepatic antioxidant enzymes, except Cu,Zn-SOD, showed a significant alteration in activity in response to exercise. Hepatic Cu,Zn-SOD activity was significantly increased following exhaustive exercise. Ji and Fu (1992) concluded that exhaustive exercise imposes a severe

oxidative stress on skeletal muscle in which the glutathione system and antioxidant enzymes are important coping mechanisms. Similarly, Jenkins *et al.* (1984) measured catalase and SOD levels in biopsies of human vastus lateralis muscle and reported significant correlations between the activities of both enzymes and VO_{2max}, an indicator of aerobic capacity. The results of a study by Jenkins (1988) indicate that the glutathione system of skeletal muscle adapts well to exercise and training. He reported an increase in glutathione synthetase in the muscles of endurance trained dogs and higher glutathione levels in trained versus untrained dogs. Furthermore, endurance trained rats were found to exhibit less erythrocyte hemolysis (perhaps due to a decreased rate of lipid peroxidation) and an increase in activities of SOD and glutathione peroxidase. Powers *et al.* (1994), Quintanilha (1984), and Kanter *et al.* (1985) have also found increased SOD activity with training. Gohil *et al.* (1988) found a 60% decrease in glutathione and a 100% decrease in oxidized glutathione (GSSG) in whole blood during prolonged submaximal exercise. Robertson *et al.* (1991) found that the activity of the erythrocyte enzymes, catalase and glutathione peroxidase were significantly correlated with weekly training distance of human subjects. They also reported an increase in total erythrocyte glutathione in the trained subjects which was accounted for by an increase in the reduced form of glutathione.

However these findings are in contrast with those of Alessio and Goldfarb (1988) who found that total SOD activity was not affected by either acute or chronic exercise. Studying rats, Higuchi *et al.* (1985) found no increase in Cu,Zn-SOD, but a 37% increase in fast and slow twitch muscles and a 14% increase in white muscle. These increases were accompanied by a two fold increase in those constituents of the mitochondrial respiratory chain responsible for the production of superoxide. Thus, these researchers concluded that the relatively small increase in the mitochondrial SOD activity by endurance training was inadequate in protecting

against free radical damage and hence exercise-trained muscle is prone to accelerated aging. Similarly, Ohno *et al.* (1992) in a study conducted on 31 healthy male medical students (12 sedentary and 19 long-distance cross-country skiers aged 19-24) found only a significant elevation of plasma Mn-SOD levels in athletes as compared to sedentary individuals. No significant difference was detected in Cu,Zn-SOD levels between the two groups. Ohno *et al.* (1992) suggested that Mn-SOD concentration is a reliable index of physical training. Duthie *et al.* (1990) assayed erythrocyte SOD, catalase and glutathione peroxidase activities of seven trained athletes after the completion of a half-marathon and found no change in these enzymes.

The discrepancies in the literature regarding the response of the antioxidant enzymes, such as SOD, to exercise are perhaps due to differences in training protocol used by the investigators (e.g., intensity of exercise), tissues examined, and time of assay. It is possible that physical activity must be of a certain minimum intensity before it acts as a sufficient stimulus to promote upregulation of SOD activity. Similarly, differences may exist in the response of antioxidant enzymes to exercise in terms of short-term and long-term adaptations. Hence, measurement of antioxidant enzymes during, immediately following, shortly after or much after a training regime may yield different and conflicting results. In the present study SOD was assayed via a continuous multiple point line rather than the single point assay which other researchers are using to determine SOD activity. The multiple point assay in which approximately five points are collected in duplicate seems to be a more accurate way of determining enzyme activities. Furthermore, in this study a physical activity index was calculated in kcal/wk. An estimation of physical activity levels in kcal/wk provides a good indication of a person's overall habitual activity levels and does not arbitrarily assign a person as active or inactive based on limited, highly specific information such as participation in a particular sport (e.g., long distance cross-country skiing). Such limited information is

inadequate in providing an indication of overall activity levels. Determination of kcal/wk of energy expenditure is an objective way of comparing overall activity levels within a given population and may be a useful index in terms of allowing direct comparisons of human physical activity level studies between different labs.

Influence of exercise on SOD activity

In this study it was found that exercise was beneficial regardless of age or gender, as active individuals in each age category and both males and females displayed higher SOD activity than their inactive counterparts. These findings are also in agreement with research investigating the effects of exercise in the context of aging. Kretzschmar *et al.* (1993) found that the age-dependent decline in plasma glutathione levels could be partly compensated for by physical training. In an earlier publication by Kretzschmar *et al.* (1991), it was reported that well-trained individuals aged 36-57 years had a higher reduced glutathione level in circulating blood than did untrained individuals of the same age. Therefore, exercise training was found to be an effective means of restoring the age-dependent decline in plasma glutathione and in enhancing resistance to oxidative stress. Thus, endurance training was found to be beneficial in improving the body's antioxidant defence capacity.

In agreement with the trends seen in the present work, Lammi-Keefe *et al.* (1984) found an increase in SOD activity in skeletal and heart muscles of exercised rats with an increase in age of up to 23 months. Ji *et al.* (1990) reported that following exhaustive exercise there were increased activities of several enzymes involved in oxygen free radical metabolism in skeletal muscle with age, including the enzymes SOD, catalase and glutathione peroxidase. Also, the activities of glutathione-S-transferase, glutathione reductase and glucose-6-phosphate dehydrogenase, which are supporting enzymes in the cellular antioxidant function, were also higher in middle-aged and senescent rats compared to young rats. Exercise/training induced

upregulation of antioxidant enzymes if persistent over extended periods of time may be desireable in terms of compensating for the age-related declines in these enzymes. Free radical damage has been linked with the aging process as the damage inflicted by these highly reactive species at the molecular level, can effect every aspect of the body. This tends to promote accumulation of damage, senescence and eventually death.

Our results are in disagreement with those of Ji, Wu and Thomas (1991) who were the first to study the response of antioxidant enzymes to training in senescent animals. They found no change in rat skeletal muscle SOD or catalase activities following a 10 week endurance training program. Glutathione activity however was found to increase dramatically (62%) in the deep portion of the vastus lateralis muscle in old rats following training. Moreover, glutathione peroxidase induction was comparable to that observed in young trained rats. Kretzschmar *et al.* (1991) also reported an age-dependent decrease in plasma glutathione levels which could be partially compensated for by training. They suggested that increased export of glutathione from skeletal muscle into circulation was responsible for this effect.

Nonetheless, an adaptive response by the antioxidant system to exercise seems very reasonable as an intrinsic protective mechanism. Direct benefits of antioxidants have been demonstrated by studies in which human subjects or experimental animals are given antioxidant supplements. Although Zerba *et al.* (1990) did not measure muscle SOD activity, they found that pretreatment of old mice with SOD provided immediate protection against injury. Also, a study by Cannon *et al.* (1990) revealed that a vitamin E supplementation of 800 IU per day for two months prior to exercise significantly increased the post-exercise rise in circulating neutrophils and creatine kinase activity in older subjects (>55 years) only. Similarly, Meydani *et al.* (1990) found that following exercise the excretion of lipid peroxides in urine increased in both the young (<30 years) and old (>55 years) age groups; however, vitamin E

supplemented subjects excreted a lower level of these compounds. These results suggest that vitamin E supplementation can suppress the oxidative damage induced by exercise.

The accumulated data seems optimistic with respect to attenuating some of the antioxidant declines observed in aging individuals. Thus, endurance training can potentially have a great impact on preventing the declines in antioxidant and metabolic functions associated with senescence and needs to be further explored.

Influence of gender on SOD activity

In the present study SOD activity was found to show some sex differences, as the mean values for SOD activity in males tended to be higher than those for females for each of the age and physical activity subcategories. This study appears to be the first to have looked at the relationship between age, antioxidant capacity, activity levels and gender. Literature on the relationship between health-related parameters and exercise in females is virtually nonexistent. Exercise studies clearly have concentrated mainly on men and gender differences have been neglected. The lack of data involving females is reflected by the absence of women in studies conducted by Paffenbarger *et al.* (1993), Sandvik *et al.* (1993) and most others. In this study it was found that the mean SOD activity in males was 7% higher than in females (difference not significant). Males were also found to have a 16% higher concentration of Lowry extracted protein than females (difference in means not significant). Also, males had a 14% higher hemoglobin concentration in comparison to females ($p=0.0001$). This agrees with the findings of Aliakbar *et al.* (1993) in which males were found to have higher concentrations of SOD enzyme normalized with respect to hemoglobin, when compared to females (652 ug SOD/g Hb, males; 635 ug SOD/g Hb females). The differences found in this study between males and females and active and inactive individuals may be a reflection of differences in metabolic rate within these groups.

Exercise benefits in females and the elderly

In this study it was noticed that exercise seems to generally have a proportionally greater benefit, in terms of increased SOD activity levels, in older vs younger people and in females compared to males. This may be due to the fact that the SOD activity in young individuals and in males is already at a more optimal level than that of older individuals or females. The finding that the exercise benefits are more pronounced in the aged is further supported by the findings of Cannon *et al.* (1990), who showed that the positive effects of vitamin E supplementation on neutrophil response following exercise were more dramatic in the older subjects (>55 years) than the younger subjects (<30 years). Furthermore, Meydani *et al.* (1990) found that the decreased excretion of lipid peroxides in the urine of young (<30 years) and old (>55 years) subjects following exercise, upon vitamin E supplementation, was more prominent in the older group (i.e., the protective effect of vitamin E was greater in the older subjects).

Putative factors responsible for increased SOD activity

Once it was established that SOD activity levels were changing, it was important to resolve what was effecting this change. The increased SOD activity must be attributable to either an increased concentration of the enzyme (Et) and/or to changes in the intrinsic physical properties of the enzyme (kcat or Km). The cause of the increased SOD activity can be isolated by considering the Lowry protein and temperature data.

A plot of SOD activity vs extract Lowry protein concentration yields a linear graph ($p=0.0001$). Thus, SOD content appears to be a constant fraction of the Lowry extracted protein (i.e., it is proportional to the purity of the extract) (Fridovich, personal communications). Hence the slope of this graph is proportional to SOD activity. The slope of this graph can be affected by three factors: (1) changes in SOD concentration due to

altered gene expression or stability of the enzyme, (2) changes in SOD physical properties (e.g. energy of activation, Ea), and (3) changes in protein concentration in the extract. An increase in SOD concentration, a decrease in Ea of the SOD enzyme, and a decrease in total protein concentration can all lead to an increase in the slope of this graph and vise versa. Thus, these factors must be accounted for in order to determine why SOD activity is higher in the active and younger individuals.

An indication of the effect of Lowry protein on the magnitude of SOD activity can be obtained from an analysis of the mean value of total Lowry protein in the inactive and active groups. Lowry protein is found to be higher in the active group (all individuals expending >1500 kcal/wk) as compared to the inactive group (all individual expending < 1500 kcal/wk) by approximately 7%. This rules out the possibility that a drop in Lowry protein is leading to an increased slope for the linear plot of SOD activity vs Lowry extracted protein. In fact, the higher levels of total protein detected in the active and young groups would have the opposite effect on this parameter compared to elevated SOD activity due to increased SOD concentrations and/or decreased Ea, which both tend to increase the slope.

The mean value of SOD activity is also higher in the active group as compared to the inactive group by approximately 39%. This increased activity can only be influenced by two factors: increased amount of the enzyme and/or decreased Ea. An increased amount of SOD can be due either to an accelerated rate of synthesis or a decelerated rate of degradation of the protein. The temperature study in which SOD was assayed at both 22 and 40 degrees Celsius to determine relative catalytic efficiency was useful in separating out changes in total amount of SOD from physical property changes. The Arrhenius plot provides an indication of enthalpy of activation which is an indicator of physical property changes of the enzyme. A statistical analysis of the relationship between ln temp and SOD activity showed no

correlation. Hence, it can be inferred that the increase in SOD activity must be due to increased levels of SOD enzyme.

From this, one can conclude that either gene expression increases or SOD degradation rate decreases under conditions of chronically elevated physical activities as an adaptive response to deal with the increased concentration of oxygen free radicals. Similarly, it can be inferred from the results that SOD gene expression decreases or SOD degradation rate increases over the course of a lifetime, thereby subjecting the body's cells to increased oxidative stress. Maintaining relatively high physical activity levels can compensate partly for some of this loss and it has even been found to be beneficial to senescent individuals. The induction of SOD via increased synthesis of SOD agrees with the findings of Ji *et al.* (1991) who showed that even senescent rats could elevate antioxidant enzyme levels through physical training. Although aging may significantly affect antioxidant and metabolic capacities in skeletal muscle, regular exercise can preserve functions of these enzymes even in old age (Ji *et al.*, 1991). Induction of SOD is consistent with the findings of other researchers. Control of SOD-1 activity at the pretranslational level of gene expression has been documented in both rats and mice (Delabar *et al.*, 1987). Furthermore, Gregory and Fridovich (1973a) have reported induction of SOD by oxygen stress in aerobic bacteria. Also, Jenkins *et al.* (1984) reported increased Cu,Zn-SOD activity after training in human skeletal muscle and Ji *et al.* (1992) found that muscle tissue can up-regulate the activity of both Cu,Zn-SOD and Mn-SOD during aging. However, Halliwell and Gutteridge (1984) found that only Mn-SOD responded to increased exposure to molecular oxygen in various organs and tissues. These observations may have been due to experimental conditions in which the oxygen tension was sufficient to induce the Mn-SOD enzyme, but not the Cu,Zn-SOD enzyme. Thus, the threshold for enzyme induction may be higher for the cytosolic enzyme as compared to the

mitochondrial one. Ji *et al.* (1991) noted that the decrease in muscle enzyme activity in senescent rats and the corresponding increase with training were associated with parallel changes in protein content. Ji, Wu, and Thomas (1991) found that in old rats there was a significant correlation between mitochondrial citrate synthase activity and muscle protein content. Muscle protein also correlated with malate dehydrogenase activity. These findings suggest that the observed training induction of these two enzymes was due to an increase in TCA cycle enzymes. This implies that muscle mitochondrial proteins, rather than specific enzyme activities, decline with age.

However, this does not preclude the possibility that a decrease in the rate of SOD degradation may be responsible for elevated SOD levels. Oxidatively modified proteins are known to be more rapidly and selectively degraded by intracellular proteolytic systems. This may be the case in the elderly or inactive populations, where concentrations or activities of antioxidants may not be as optimal as that in the young or active groups. The decline in muscle mass and functional capacity with advancing age, in combination with decreased biochemical defense systems, may make the elderly prone to oxidative stress and injury. Exercise can slow down the rate of loss of lean body mass and functional capacity thereby decreasing the vulnerability of the elderly to oxidative damage.

Moreover, enzyme activity may not reflect the true amount of the enzyme, but rather the presence or absence of cofactors, activators and inhibitors (Ohno *et al.*, 1992). That is, increased SOD activity may be due to increased gene expression and/or allosteric modification. Jabusch *et al.* (1980) have shown that alkylation of the free SH group of human Cu,Zn-SOD increases the stability of the enzyme. This is a change in a physical property of the SOD enzyme and may or may not effect kcat/Km. The activities of glucose-6-phosphate dehydrogenase and glutathione peroxidase have been shown to decrease due to allosteric

modification through activated oxygen. Also, since active SOD-1 contains both copper and zinc, decreased SOD activity may reflect a copper or zinc deficiency.

Extract protein concentration as an indicator of oxidative stress

Another interesting finding of this study is that Lowry extractable protein can be used as an index of cellular oxidative stress. This discovery stems from a plot of Lowry protein against age and physical activity. This bar graph clearly indicates that more protein is lost with advancing age. This is likely a reflection of cellular oxidative stress which is possibly leading to protein-protein cross-linking or less stable proteins. Free radical dependent cross-linking of proteins has been reported by Nagy and Nagy (1980). These proteins experience greater difficulty surviving the harsh chloroform/ethanol extraction procedure employed in the purification of SOD from red blood cells. An SDS gel electrophoresis of a sampling of two subjects detected only four other proteins besides SOD in the chloroform/ethanol extracts. These had masses of 63, 54, 35, and 14 kDa and were tentatively assigned as glutathione reductase, catalase, glyceraldehyde-3 phosphate dehydrogenase and calmodulin respectively. Changes in these proteins are likely a reflection of the overall changes occurring to general proteins within the red blood cell. It was estimated that SOD constitutes approximately 1.7% of the proteins in the chloroform/ethanol extracts of active individuals and it makes up about 1.3% of the total protein in the extracts of inactive subjects. From this information and also the following facts: (1) a 39% higher SOD activity in the active versus inactive group ($p=0.0003$) and (2) a 7% greater concentration of Lowry extractable proteins in the active versus inactive group (difference not statistically significant), it appears that the difference in Lowry extracted protein and SOD activity between the two groups can be attributed to enhanced cellular protection by increased SOD concentrations. 0.3% (1.7%/1.3%) of the 7% difference in Lowry extracted protein is accounted for by differences in the total amount of

SOD. Thus, the majority of the difference in Lowry extracted protein between the two groups can be explained by a protective effect of the elevated SOD on the stability of the cell proteins (of which catalase, calmodulin, glyceraldehyde-3 phosphate dehydrogenase and glutathione reductase are representative). Hence, the elevated SOD levels in active vs inactive people must be sufficient to combat the increased oxidative stress associated with exercise and it may also lead to a slight (~7%) protective effect over the long term. Within each age category the difference between active and inactive individuals of a particular age bracket was found to be insignificant. This suggests that the elevated SOD activity which accompanies exercise is only sufficient to combat the increased oxidative stress associated with greater oxygen flux, but does not combat the total effects of aging. It is speculated that the increase in SOD concentration in active individuals is proportional to the increase in basal metabolic rate of these people in comparison to same age inactive controls. This has a protective effect as active people were found to have higher concentrations of Lowry recovered proteins.

Relationship between SOD activity and metabolic rate

From this study it seems that SOD concentration may parallel changes in metabolic rate. Normal aging is accompanied by a gradual decline in metabolic rate. This is reflected in parameters related to metabolism such as caloric consumption and VO_{2max} (Fleg and Goldberg, 1990). Reports indicate that resting metabolic rate decreases as a function of age and that VO_{2max} decreases to approximately 50% over the whole lifespan. The decline in metabolic rate correlates with a decrease in fat free weight (Poehlman *et al.*, 1992). This 50% drop in metabolic rate corresponds with a similar 50% decline in SOD activity between the youngest (0-20 years) and oldest (80-100 years) groups used in the present study (when comparing groups of similar physical activity levels). Further support comes from the fact that the decreases in VO_{2max} witnessed with age (Fleg and Goldberg, 1990) may be attenuated by

exercise as indicated from the changes in SOD activity reported in the present study. These findings are also in agreement with those of Poehlman *et al.* (1992) who reported that when subgroups of younger and older individuals were paired for age and fat-free weight, but had different VO_{2max} values, a higher resting metabolic rate was observed in the trained younger (8%) and trained older (11%) men compared with untrained younger and older men respectively. Ji, Wu and Thomas (1991) expressed similar sentiment when they concluded that endurance training can greatly prevent the decline of muscle antioxidant and metabolic functions seen in senescence. Also, in this study it was noted that SOD activity was similar between the young groups (0-20 and 20-40 years), but significantly different from old groups (40-60, 60-80, 80-100 years). This parallels the curvilinear decline of resting metabolic rate with age in which significant declines were reported only after age 40 in men (Poehlman *et al.*, 1992). Thus, metabolic rate may reach a peak in young adulthood and then remain steady until around age 40 after which a gradual age-related decline begins.

An association between SOD activity and metabolism would not be surprising since metabolic leaks are an important source of oxygen-derived free radicals. Therefore, the assumptions that the rate of radical production is a function of oxygen flow through a tissue (Jenkins, 1988) and thus is correlated with antioxidant defenses seems highly plausible. This study attempted to examine the relationship between age, SOD activity, and physical activity. It was believed that enzyme activity may not only be changing as a function of age, but also as a function of other parameters as well. In the present study physical activity was tested as a predictor of SOD activity, since it is likely that induction of SOD activity is responsive to oxygen flux through the body. This study was successful in demonstrating that SOD activity is a function of both age and activity. These relationships can be describe by the following linear equation:

(kcat Et/Km)' = -0.000188 * Age (years) + 0.000014 * Physical Activity (kcal/wk)

The scatter in the data collected on SOD activity as a function of age and physical activity is probably due to a failure in controlling for other factors which may also be influencing SOD levels. Hence, it is imperative to know what stimuli induce SOD expression or affect it allosterically, in order to isolate and study parameters of interest.

Confounding variables

It is possible that factors such as diet (e.g., consumption of antioxidants such as vitamin E or ascorbic acid), copper or zinc deficiencies, differences in intensity of activity, genetic differences in aerobic capacity, and subclinical or undiagnosed diseases may also be effecting SOD activity. Starvation and physical exercise have been shown to increase catalase activity in mouse skeletal muscle (Ji *et al.*, 1988). Also, Powers *et al.* (1994) found that the intensity of exercise training was an important determinant in producing upregulation of rat skeletal muscle SOD and glutathione peroxidase activity. Although all subjects in our study appeared to be healthy and were not taking any medications, the possibility exists that apparently healthy people may be suffering from a subclinical or undiagnosed disease. Such selective influences cannot be eliminated entirely; however, efforts should be made to discover and control for other influences that can be isolated. This would improve the comparability of results and perhaps lead to some consensus among studies looking at aging and exercise effects on SOD activity.

The benefits of exercise

The increase in SOD activity with exercise found in this study seems to imply that exercise transiently increases free radical production such that their concentration exceeds the antioxidant capacity of the tissues. This results in transient oxidative stress which provides a stimulus for upregulation of SOD activity. In physically active people, the substantial

induction of antioxidant capacity presumably protects the individual against oxidative stress over the long-term and may attenuate some of the declines associated with aging. This is consistent with literature that promotes exercise as a means of slowing down some of the changes associated with senescence. Paffenbarger *et al.* (1993) reported a 23-29% lower risk of dying from any cause among subjects who were physically active (physical activity index > 2000 kcal/wk) compared to sedentary men (physical activity index < 2000 kcal/wk). Sandvik *et al.* (1993) found an approximately 50% lower mortality rate in the most physically fit men compared to the least physically fit men. The lower mortality was attributed mainly to a decreased risk of dying from cardiovascular causes. A recent meta-analysis of 27 studies on habitual physical activity in the primary prevention of coronary disease supports these conclusions (Berlin and Colditz, 1990).

The health benefits of a physically active lifestyle may be partly due to concomitant changes in antioxidant levels. Free radicals are currently implicated in the etiology of several diseases such as atherosclerosis, arthritis and cancer. These diseases are among the leading causes of morbidity and mortality in our society. Thus, the benefits of exercise and the role of antioxidants in protecting against free radical damage will undoubtedly be a fruitful and worthwhile area of research.

Conclusions

This study found that SOD activity is 39% higher in active individuals compared to inactive individuals. The difference in enzyme activities between active and inactive individuals holds true across the age spectrum. Thus, even elderly individuals can elevate their SOD levels by engaging in regular physical activity. This is beneficial in attenuating the ~50% loss in SOD activity which occurs over a lifespan. The higher SOD activities were associated with a 7% higher extract Lowry protein concentration in the active vs inactive population. Higher concentrations of extract Lowry protein are diagnostic of better health since, loss of Lowry protein appears to be due to labilization of RBC proteins via oxidative damage.

The increased SOD activity was attributed to an increase in the total amount of the enzyme (via increased gene expression or decreased SOD degradation), since no significant differences were noted in physical properties of the enzyme (i.e., catalytic efficiency) as a function of age, physical activity levels or gender.

SOD activity seems to be a reflection of metabolic rate. This is supported by parallel changes in SOD activity with respect to aging and exercise and the higher mean levels of SOD activity in males as compared to females. Females and elderly individuals may have the most to gain by increasing their physical activity levels, since these two groups gained the most proportionally when comparing inactive to active individuals.

Future Studies

In order to definitively answer the question of whether the increased SOD activity in active vs inactive individuals and in young vs old individuals is due to increased concentrations of the enzyme an ELISA or radioimmunoassay should be included along with a determination of SOD activity levels. Also, perhaps a more refined temperature study using the Arrhenius equation to determine Ea should be conducted in which SOD activity is assayed over a broader range of temperatures and more points are collected for each subject to better define the line ln %I = -Ea'*1/T + ln a. Also, it would be valuable to discover what the response of the other two prominent antioxidant enzymes, catalase and glutathione peroxidase, is to physical activity across the age spectrum. Furthermore, other parameters of oxidative stress should also be assayed in conjunction with a study of antioxidant enzyme adaptations to exercise. This will help pinpoint the specific advantages provided, at the molecular level, by elevated antioxidant activities.

Another major void in our understanding of the effects of exercise on well-being arises from insufficient data on the relationship between physical activity and health of women. Thus, more research needs to be done to evaluate this topic.

Table 1. Summary of Age and Physical Activity characteristics of all subject groups

Subject Groups	n	$\bar{x}$ Age (years)	$\bar{x}$ Physical Activity (kcal/wk)	Range of Physical Activity (kcal/wk)
0-20 Active Males	12	15.6 ± 2.9	7012 ± 4401	1800-17712
0-20 Active Females	5	12.2 ± 3.8	3224 ± 666	2262-4018
0-20 Inactive Females	5	12.0 ± 5.6	130 ± 288	0-644
20-40 Active Males	10	30.3 ± 5.0	3523 ± 2022	1564-8850
20-40 Active Females	10	30.6 ± 7.1	6528 ± 7208	1750-24584
20-40 Inactive Males	16	35.2 ± 5.6	579 ± 480	0-1400
20-40 Inactive Females	4	36.5 ± 2.9	429 ± 379	140-987
40-60 Active Males	11	48.9 ± 5.7	3912 ± 2548	1568-8960
40-60 Active Females	4	52.0 ± 2.9	4564 ± 2831	1736-7000
40-60 Inactive Females	9	48.6 ± 5.9	639 ± 512	0-1372
40-60 Inactive Females	10	48.3 ± 6.2	375 ± 322	0-840
60-80 Active Males	9	69.6 ± 6.2	3869 ± 1730	2184-7000
60-80 Active Females	5	70.0 ± 7.2	5592 ± 3890	3500-12510
60-80 Inactive Males	8	67.6 ± 6.9	649 ± 465	112-1386
60-80 Inactive Females	2	66.5 ± 0.7	812 ± 950	140-1484
80-100 Active Males	1	81	2600	–
80-100 Active Females	1	84	1500	–
80-100 Inactive Males	4	84.5 ± 2.6	990 ± 446	504-1442
80-100 Inactive Females	1	82	84	–

Table 2. Mean values of experimental parameters for active and inactive subjects

	ACTIVE	INACTIVE	P
(kcat Et/Km)'	0.64 (n=51)	0.46 (n=45)	0.0003
Age (years)	40 (n=69)	47 (n=58)	Not significant
Physical Activity (kcal/week)	4802 (n=69)	544 (n=58)	0.0001
Hemoglobin (g/dL)	15.4 (n=63)	15.3 (n=52)	Not significant
Extract Lowry protein (ug/mL)	6.57 (n=60)	6.16 (n=52)	Not significant
Extract Bradford protein (ug/mL)	5.67 (n=55)	5.27 (n=46)	Not significant
Arrhenius slope	-470.2 (n=49)	-395.8 (n=31)	Not significant

Table 3. Mean values of all experimental parameters for males and females

	MALES	FEMALES	P
(kcat Et/Km)'	0.57 (n=58)	0.54 (n=38)	Not significant
Age (years)	45 (n=80)	41 (n=47)	Not significant
Physical Activity (kcal/week)	2825 (n=80)	2914 (n=47)	Not significant
Hemoglobin (g/dL)	16.2 (n=69)	14.2 (n=46)	0.0001
Extract Lowry protein (ug/mL)	6.75 (n=69)	5.80 (n=43)	Not significant
Extract Bradford protein (ug/mL)	5.91 (n=58)	4.92 (n=43)	0.033
Arrhenius slope	-475.2 (n=52)	-378.5 (n=28)	Not significant

Table 4. Pearson Correlation Coefficients

	(kcat Et/Km)'	Age (years)	[Hb] (g/dL)	Physical Activity (kcal/wk)	Lowry [Protein] (ug/mL)	Bradford [Protein] (ug/mL)	Arrhenius slope
(kcat Et/Km)'	1.00 (p=0, n=96)	-0.58 (p=0.0001, n=96)	-0.11 (p=0.2722, n=96)	0.40 (0.0001, n=96)	0.82 (p=0.0001, n=94)	0.80 (p=0.0001, n=87)	0.03 (p=0.8003, n=68)
Age (years)	-0.58 (p=0.0001, n=96)	1.00 (p=0, n=129)	0.20 (p=0.0263, n=117)	-0.21 (p=0.0171, n=127)	-.33 (p=0.0004, n=114)	-0.32 (p=0.0010, n=102)	-0.04 (p=0.7420, n=80)
[Hb] (g/dL)	-0.11 (p=.2722, n=96)	0.20 (p=0.0263, n=117)	1.00 (p=0, n=117)	-0.12 (p=0.2089, n=115)	-0.05 (p=0.5876, n=113)	-0.03 (p=0.7292, n=102)	0.03 (p=0.7997, n=80)
Physical Activity (kcal/wk)	0.40 (0.0001, n=96)	-0.21 (p=0.0171, n=127)	-0.12 (p=0.2089, n=115)	1.00 (p=0, n=127)	0.20 (p=0.0314, n=112)	0.20 (p=0.0490, n=101)	0.12 (p=0.2951), n=80)
Lowry [Protein] (ug/mL)	0.82 (0.0001, n=94)	-0.33 (p=0.0004, n=114)	-0.05 (p=0.5876, n=113)	0.20 (p=0.0314, n=112)	1.00 (p=0, n=114)	0.96 (p=0.0001, n=100)	0.03 (p=0.8169, n=79)
Bradford [Protein] (ug/mL)	0.80 (p=0.0001, n=87)	-0.32 (p=0.0010, n=102)	-0.03 (p=0.7292, n=102)	0.20 (p=0.0490, n=101)	0.96 (p=0.0001, n=100)	1.00 (p=0, n=102)	0.07 (p=0.5524, n=70)
Arrhenius slope	0.03 (p=0.8003, n=68)	-0.04 (p=0.7420, n=80)	0.03 (p=0.7997, n=80)	0.12 (p=0.2951), n=80)	0.03 (p=0.8169, n=79)	0.07 (p=0.5524, n=70)	1.00 (p=0, n=80)

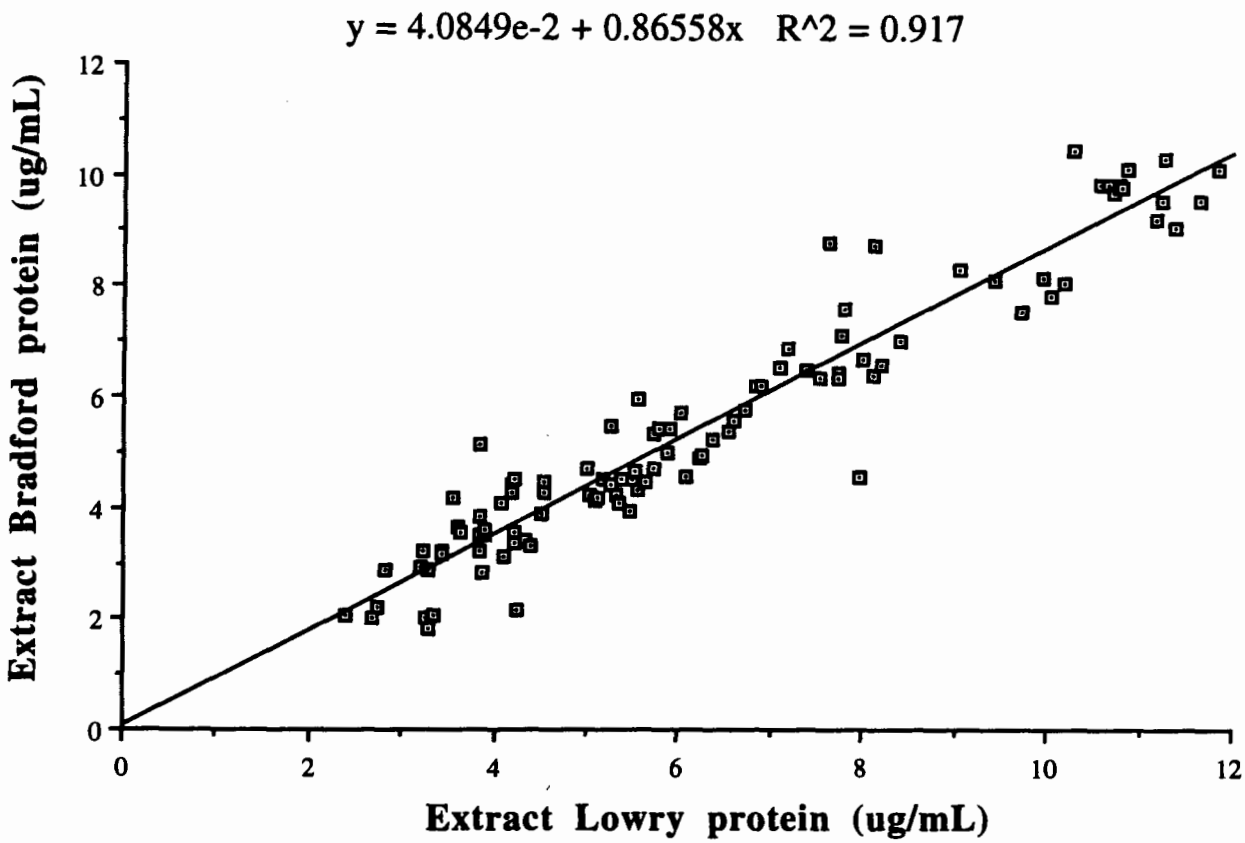

Figure 1. Correlation between protein estimation in extracts by Bradford and Lowry techniques

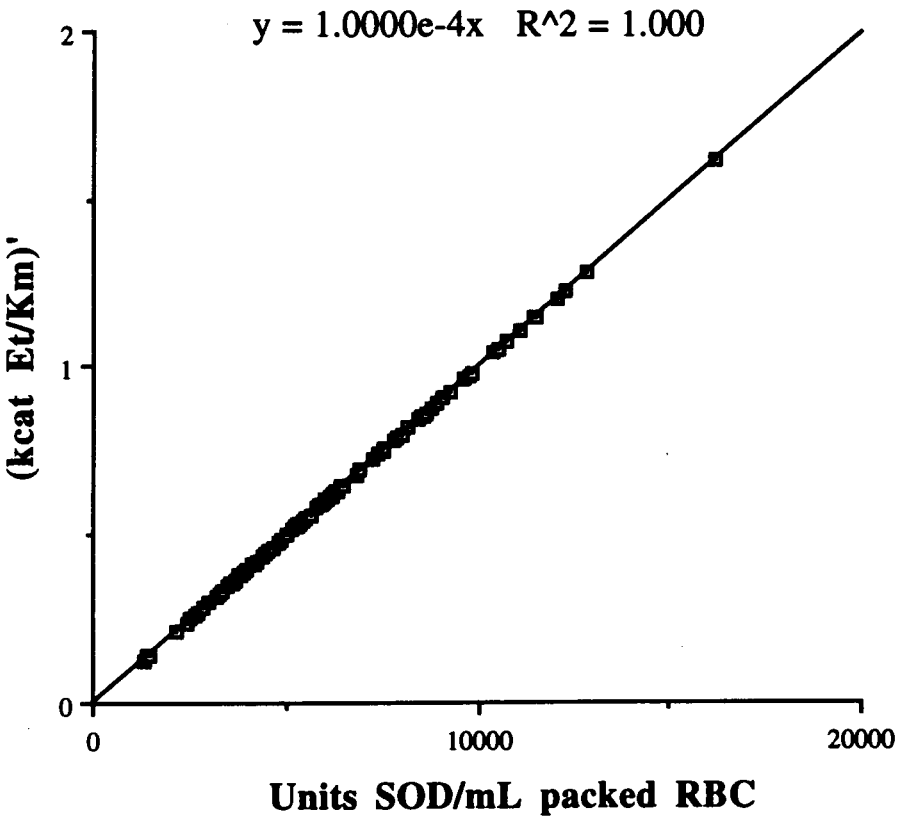

Figure 2. Relation of (kcat Et/Km)' to total SOD activity for all subjects

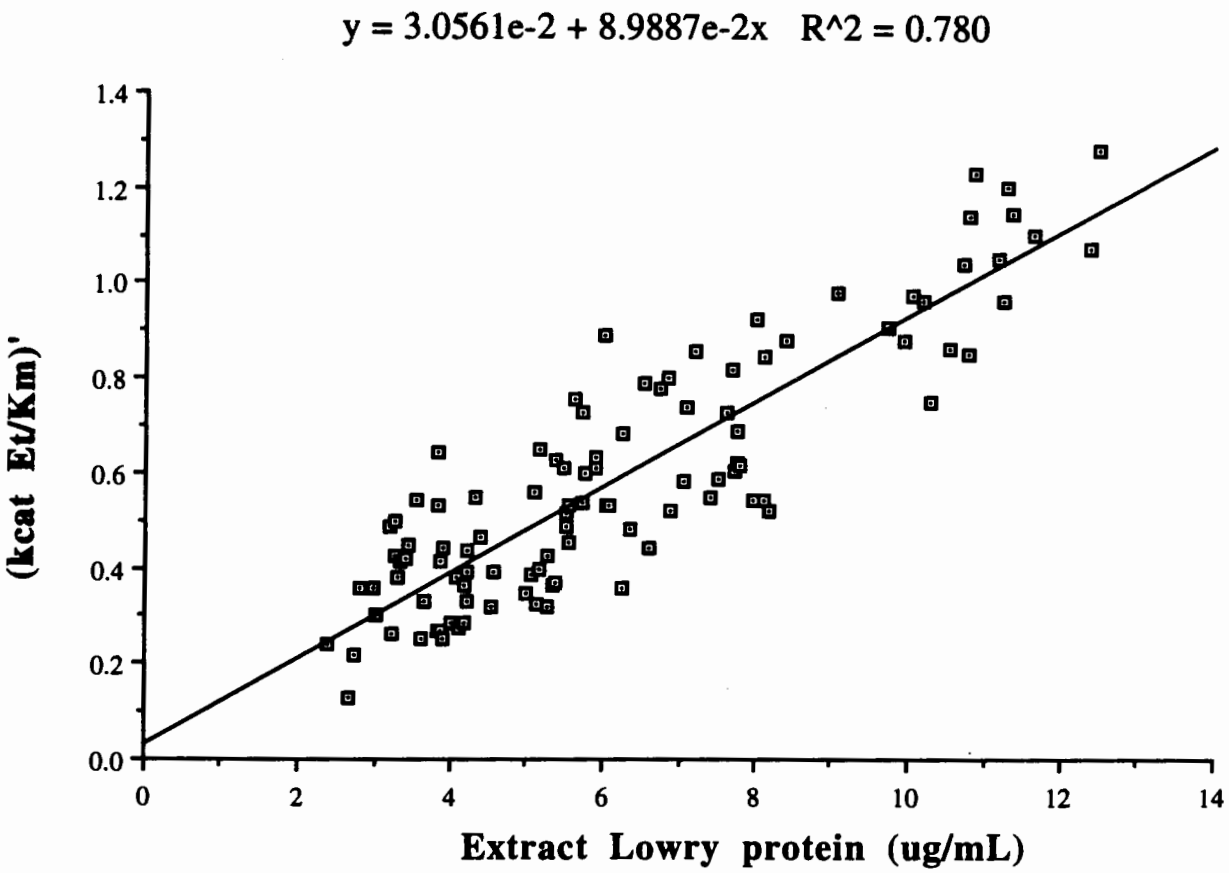

Figure 3. Relation of SOD activity to total extract protein concentration in ACTIVE and INACTIVE subjects

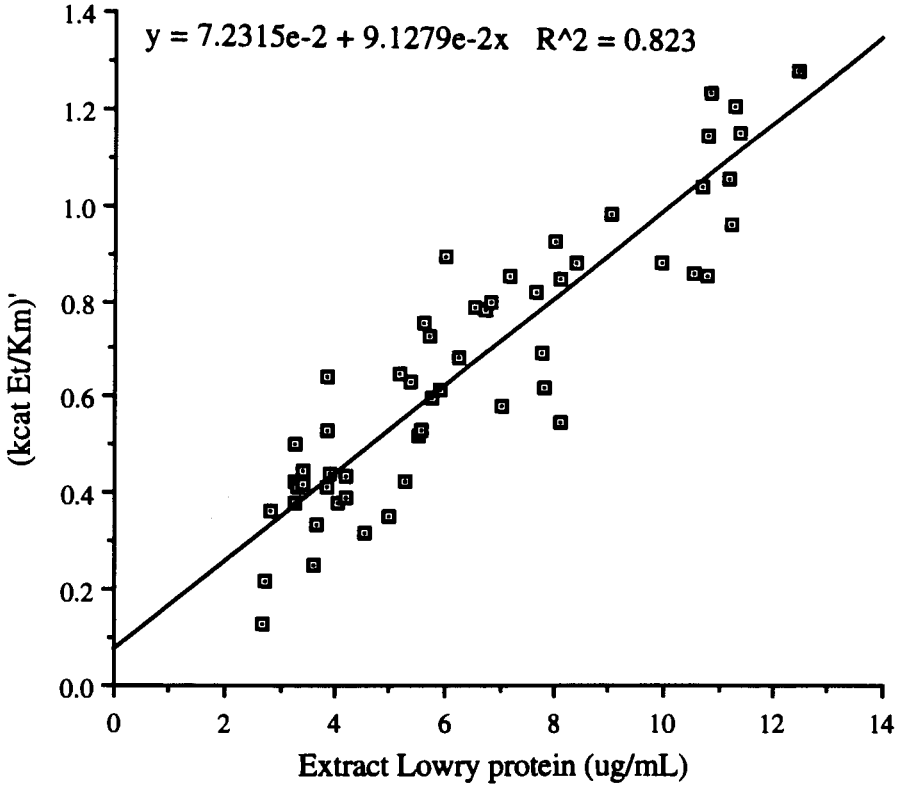

Figure 4. Relation of SOD activity to total extract protein concentration in ACTIVE subjects

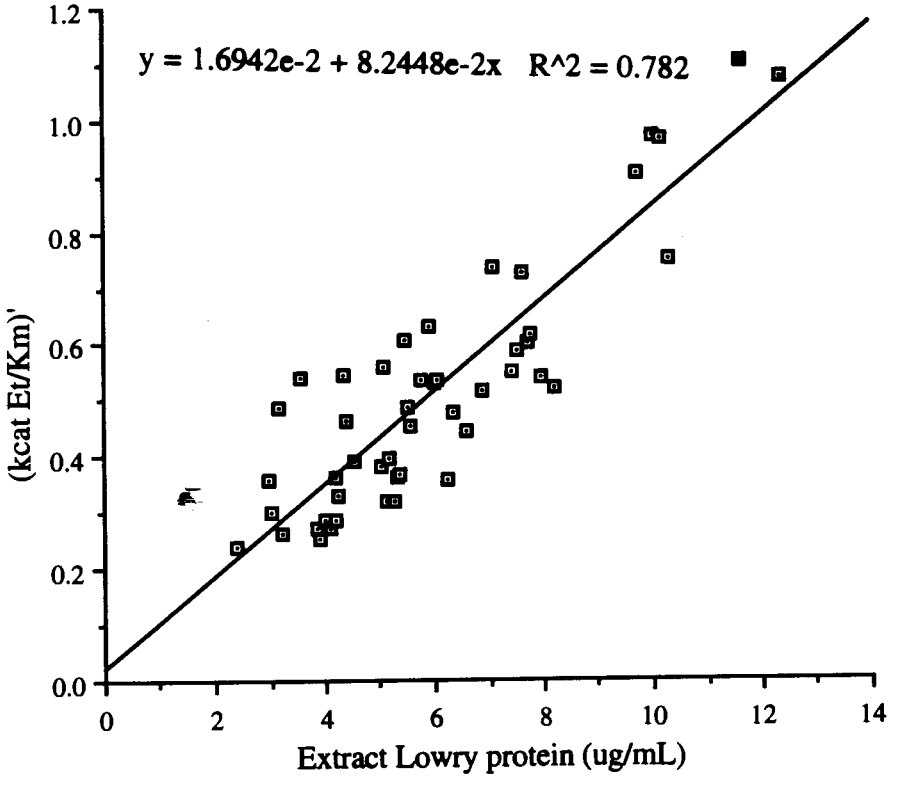

Figure 5. Relation of SOD activity to total extract protein concentration in INACTIVE subjects

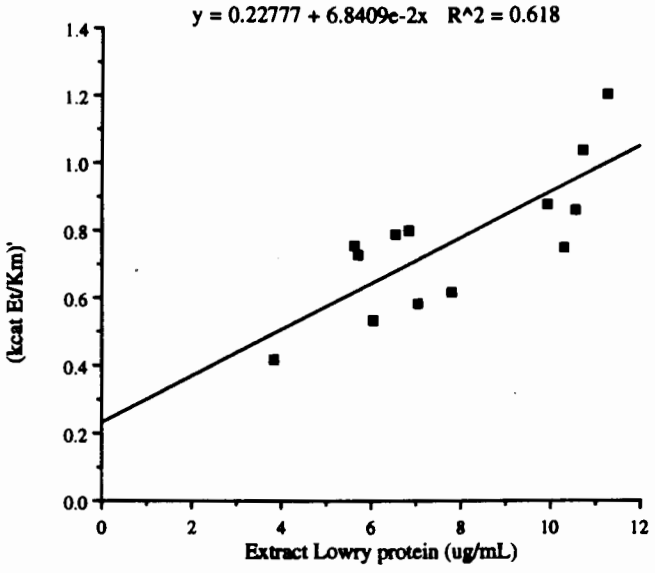

Figure 6. Relation of SOD activity to extract protein concentration in ALL subjects 0-20 years old

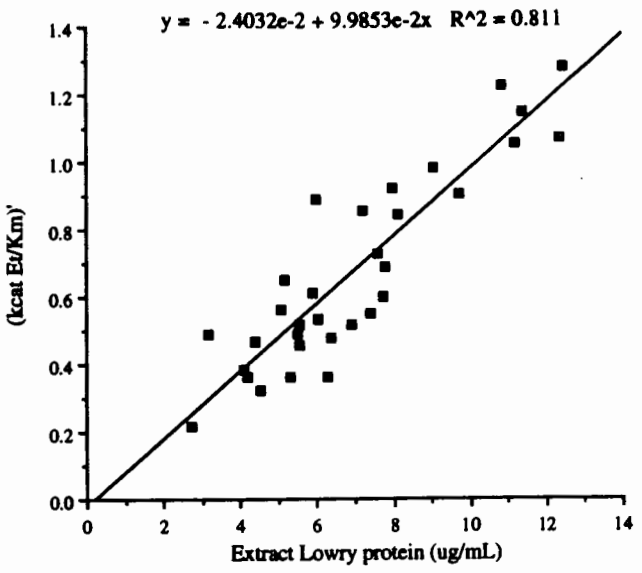

Figure 7. Relation of SOD activity to extract protein concentration in ALL subjects 20-40 years old

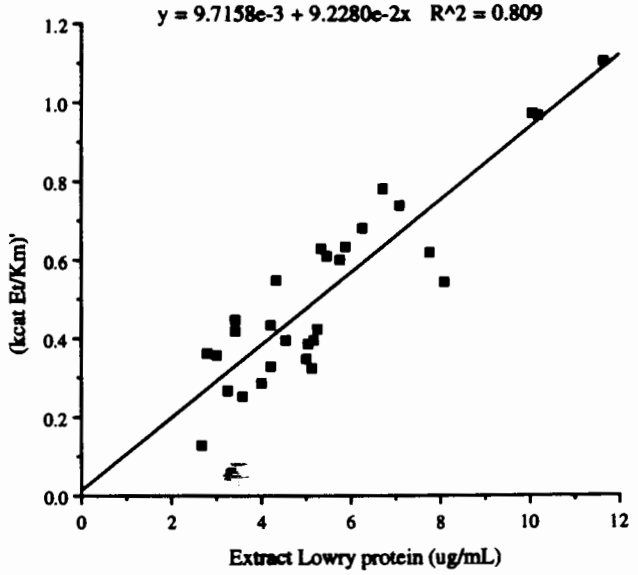

Figure 8. Relation of SOD activity to extract protein concentration in ALL subjects 40-60 years old

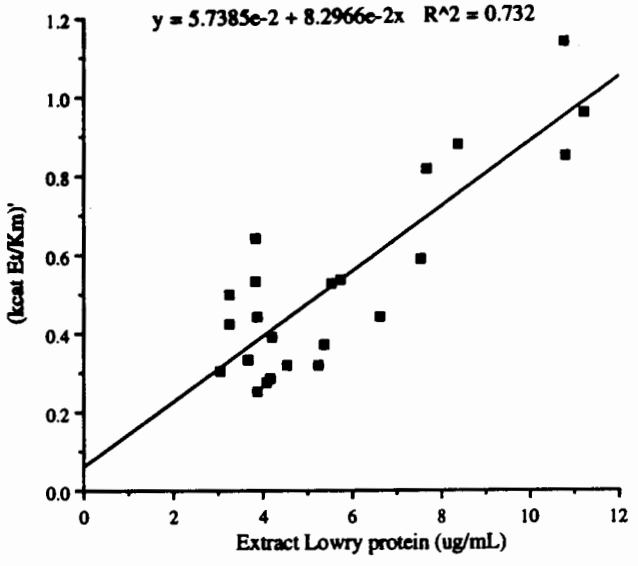

Figure 9. Relation of SOD activity to extract protein concentration in ALL subjects 60-80 years old

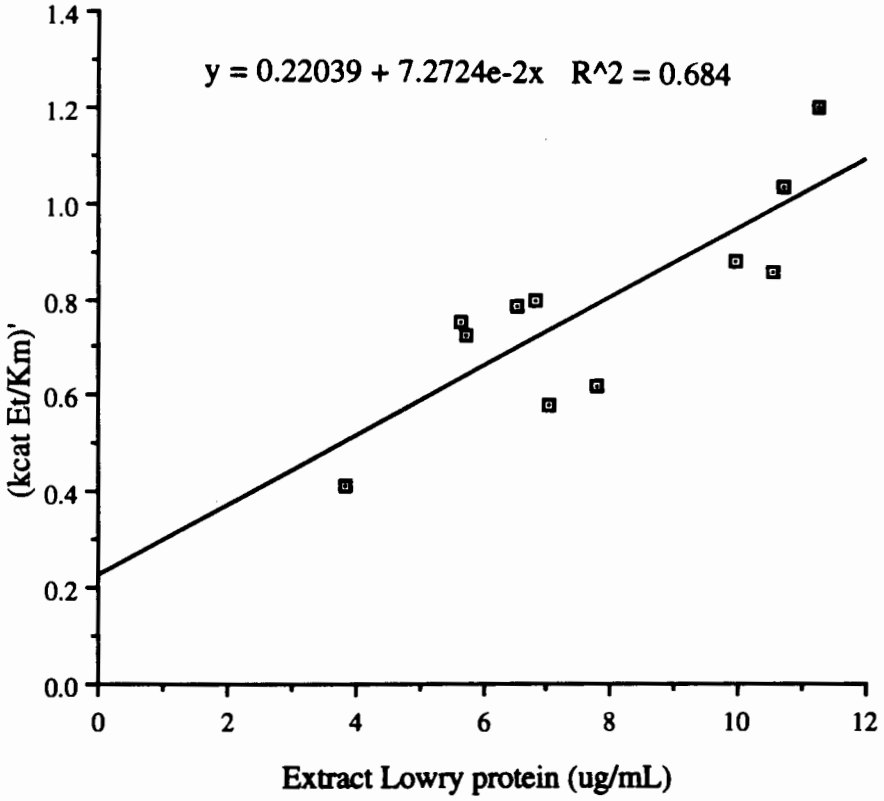

Figure 10. Relation of SOD activity to extract protein concentration in ACTIVE subjects 0-20 years old

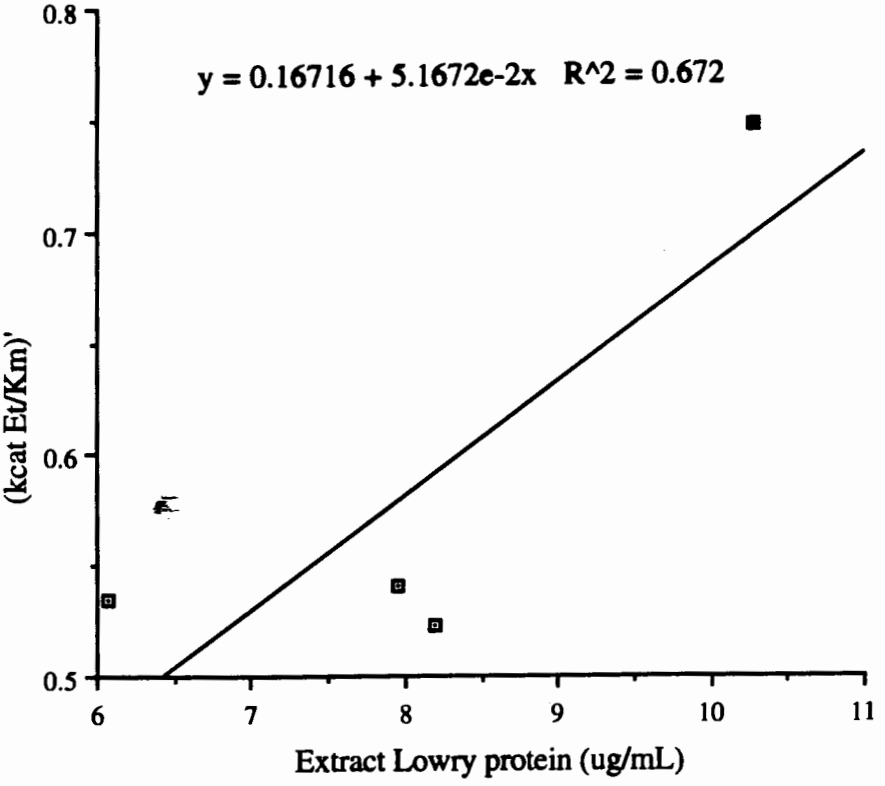

Figure 11. Relation of SOD activity to extract protein concentration in INACTIVE subjects 0-20 years old

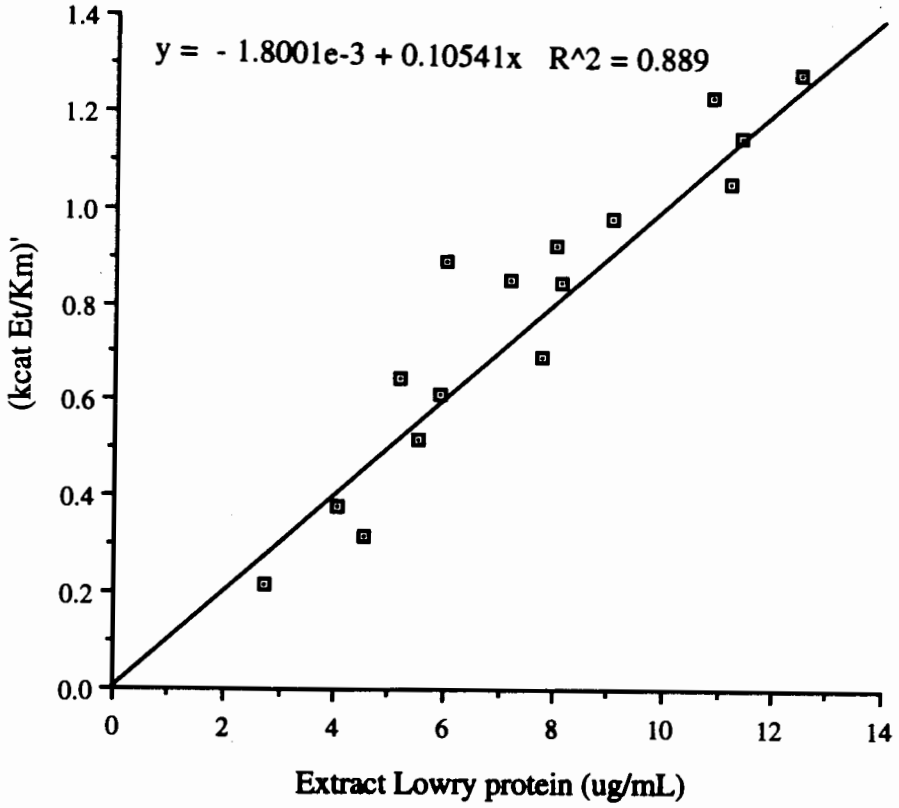

Figure 12. Relation of SOD activity to extract protein concentration in ACTIVE subjects 20-40 years old

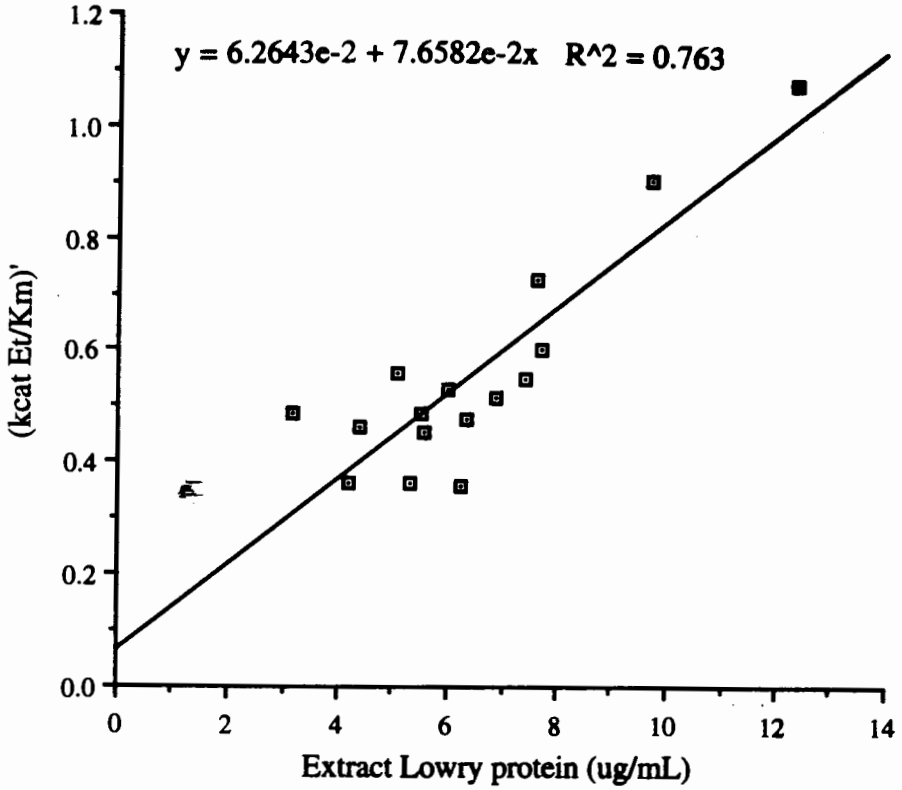

Figure 13. Relation of SOD activity to extract protein concentration in INACTIVE subjects 20-40 years old

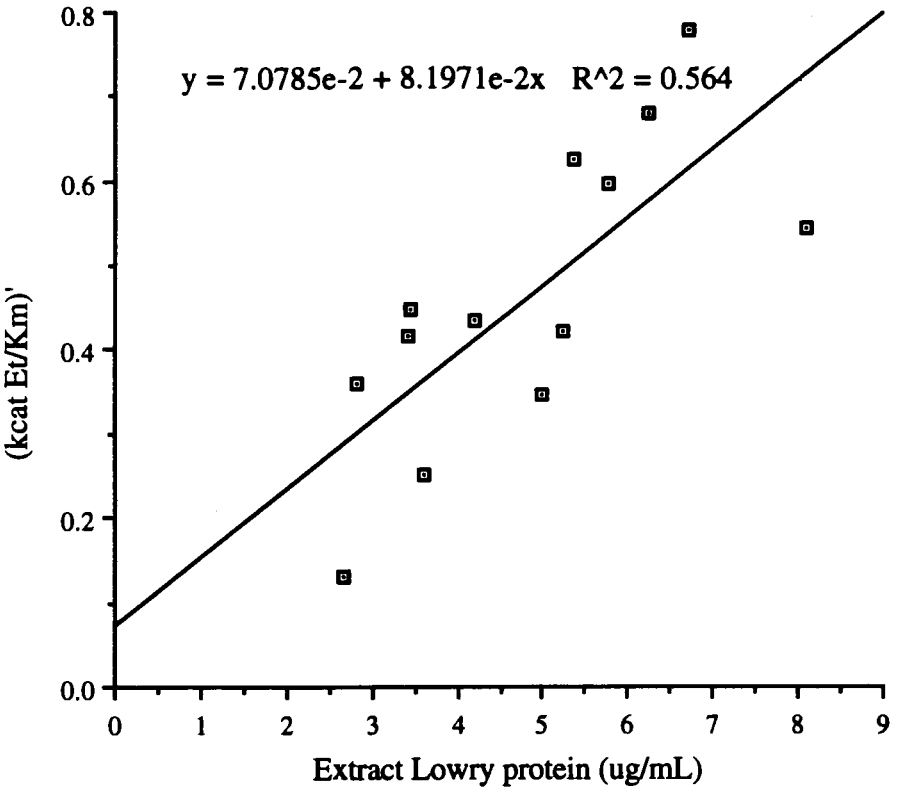

Figure 14. Relation of SOD activity to extract protein concentration in ACTIVE subjects 40-60 years old

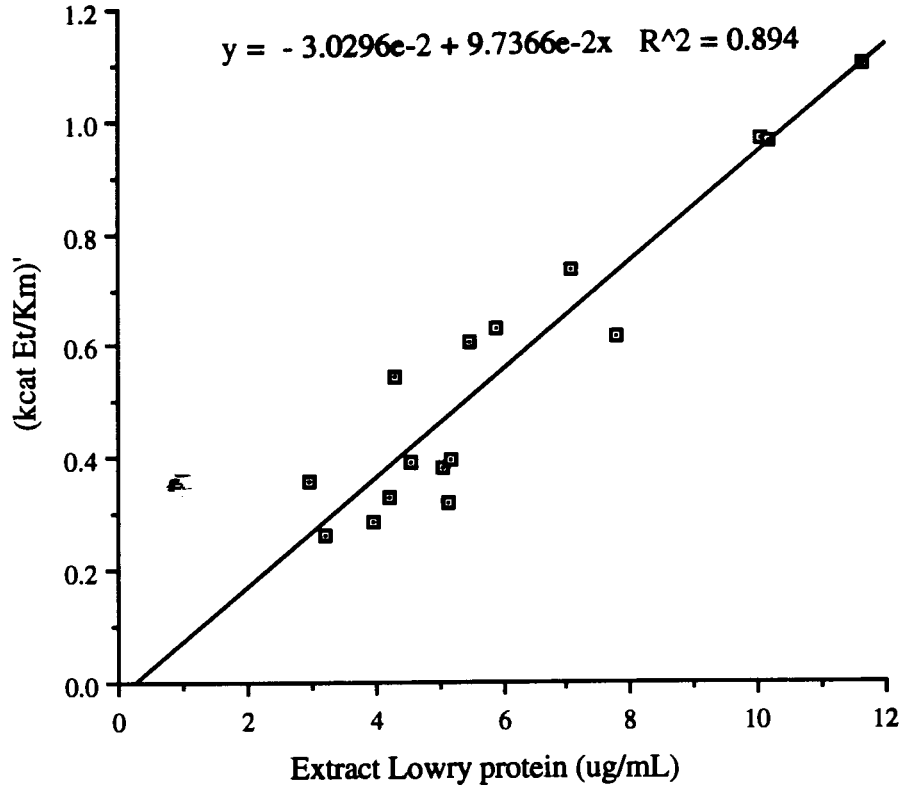

Figure 15. Relation of SOD activity to extract protein concentration in INACTIVE subjects 40-60 years old

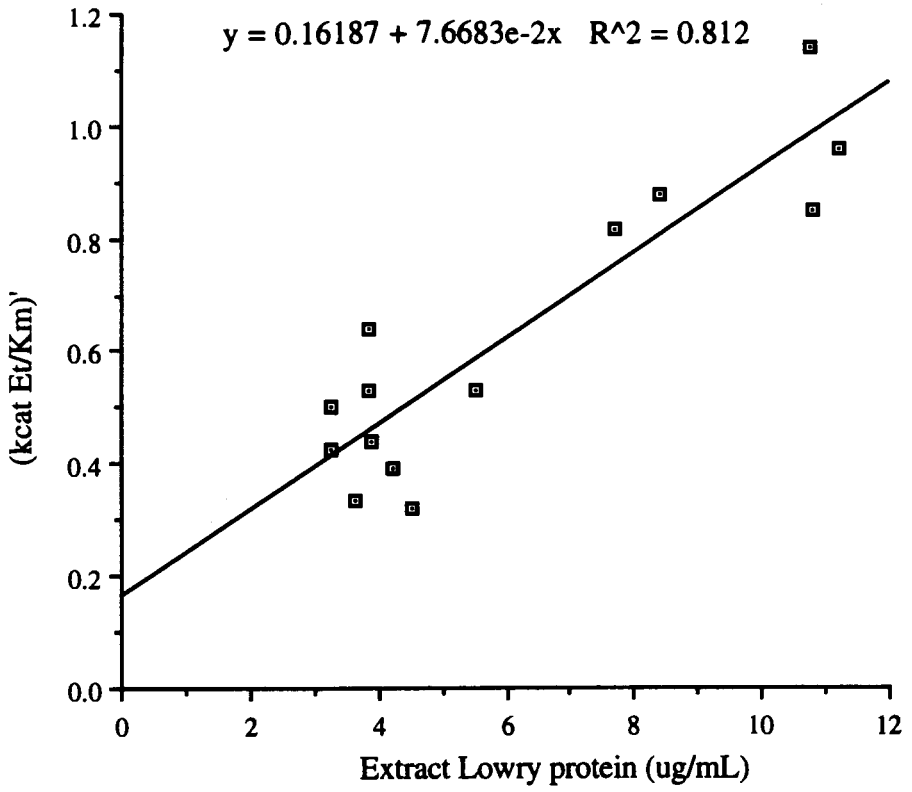

Figure 16. Relation of SOD activity to extract protein concentration in ACTIVE subjects 60-80 years old

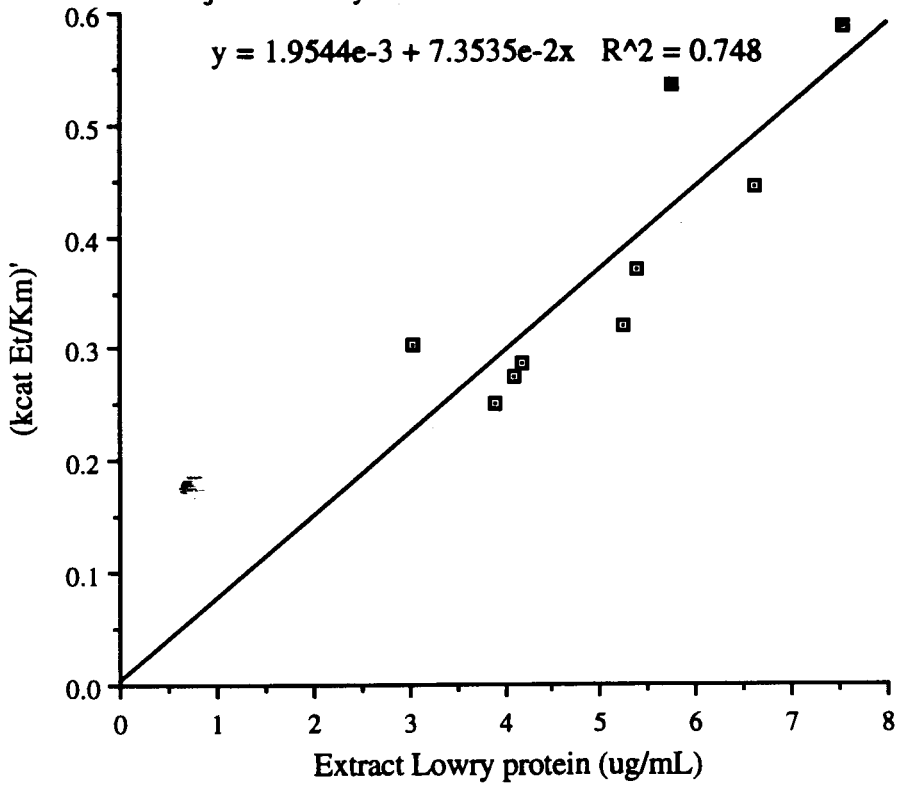

Figure 17. Relation of SOD activity to extract protein concentration in INACTIVE subjects 60-80 years old

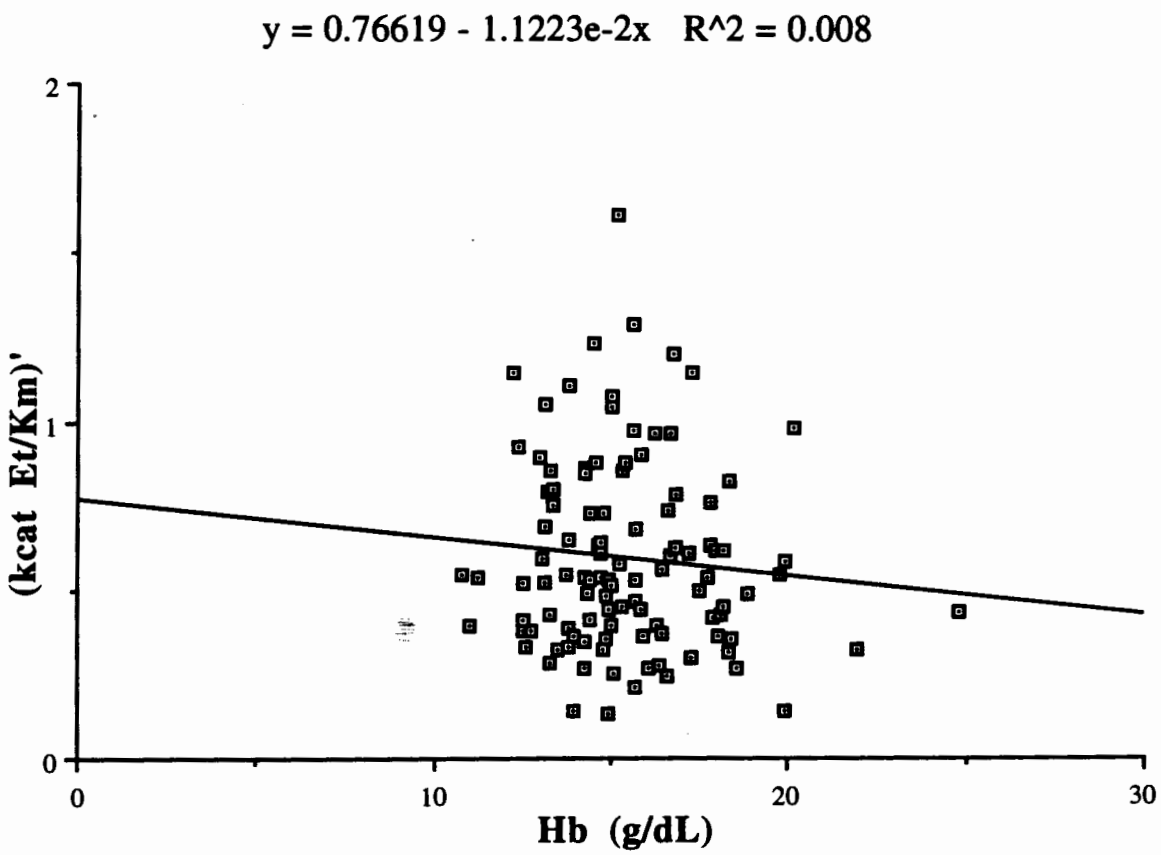

Figure 18. Relation of SOD activity to hemoglobin concentration in all subjects

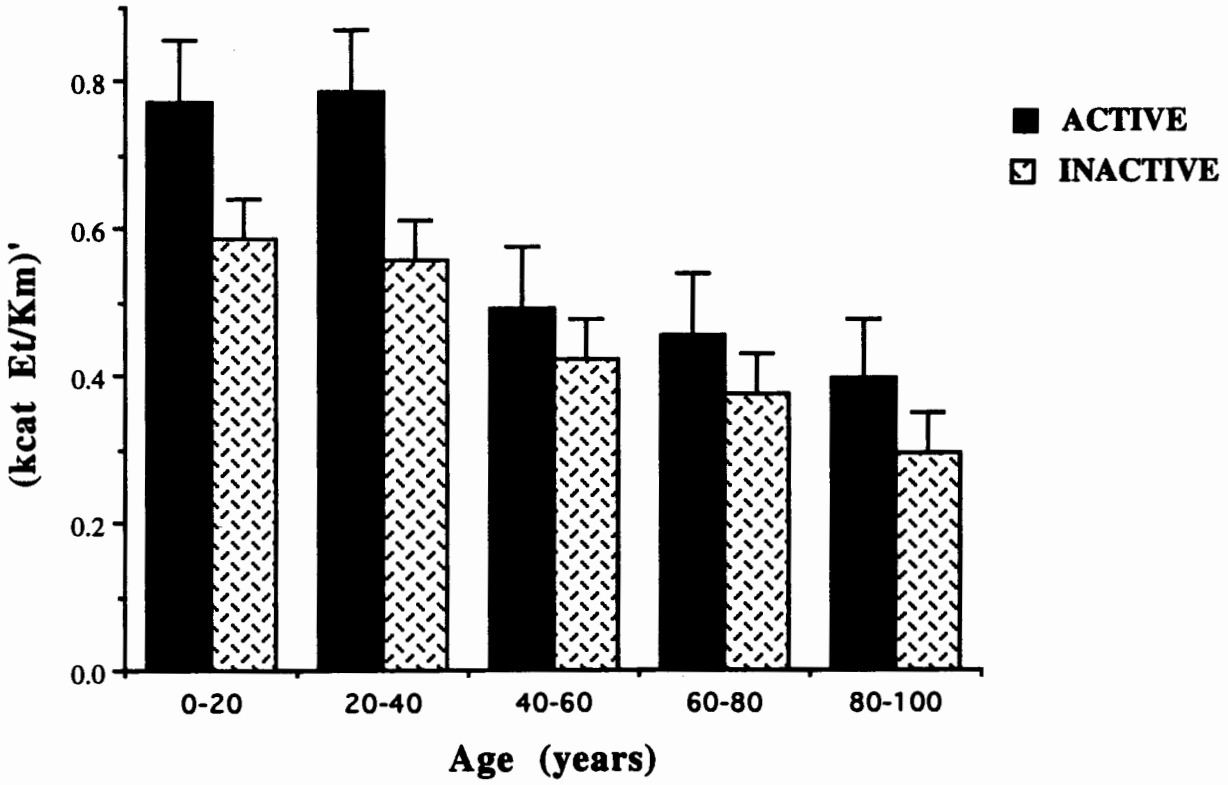

Figure 19. SOD activity as a function of age for ALL subjects separated by PHYSICAL ACTIVITY

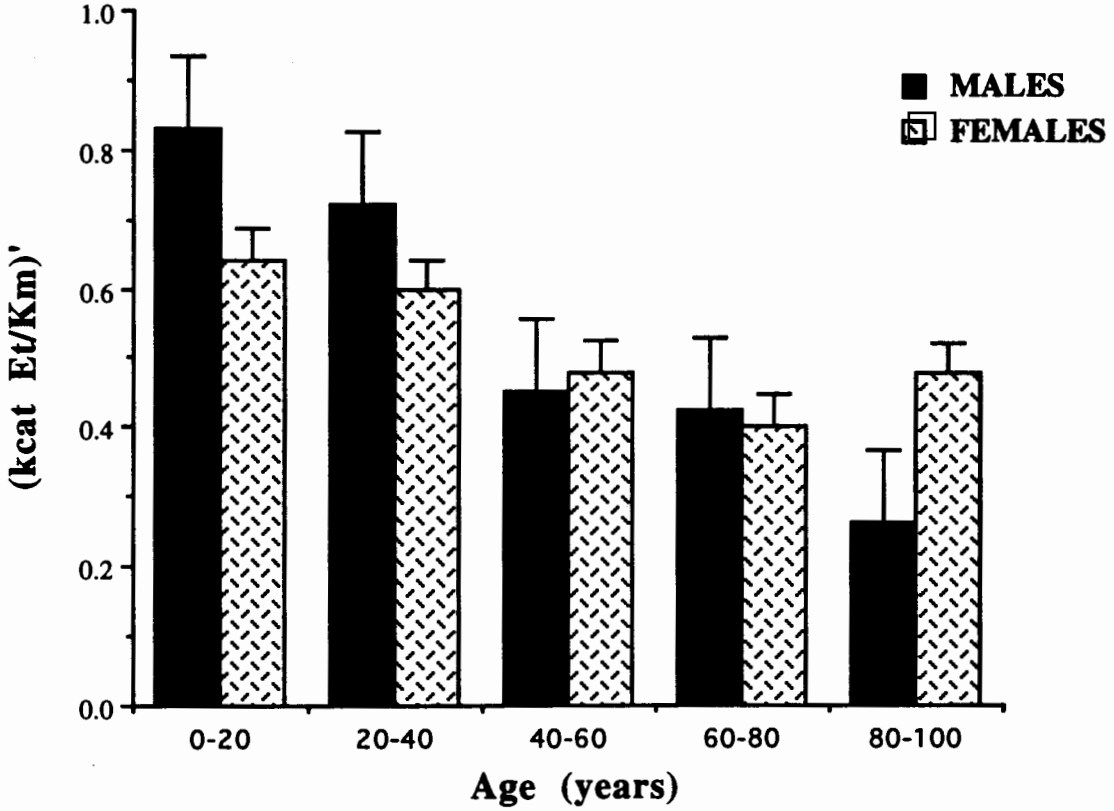

Figure 20. SOD activity as a function of age for ALL subjects separated by SEX

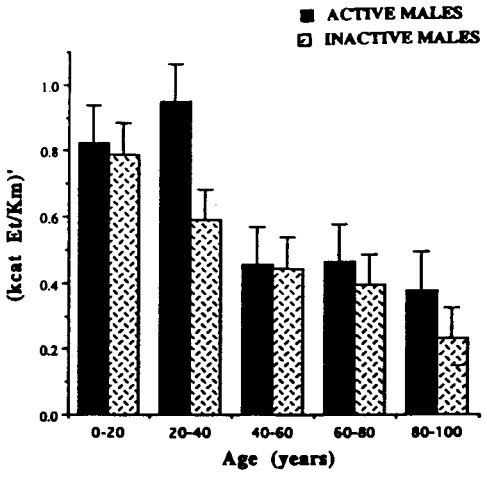

Figure 21. SOD activity as a function of age for MALE subjects separated by PHYSICAL ACTIVITY

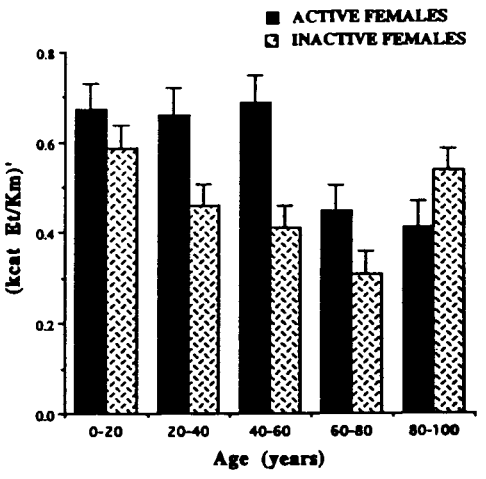

Figure 22. SOD activity as a function of age for FEMALE subjects separated by physical activity

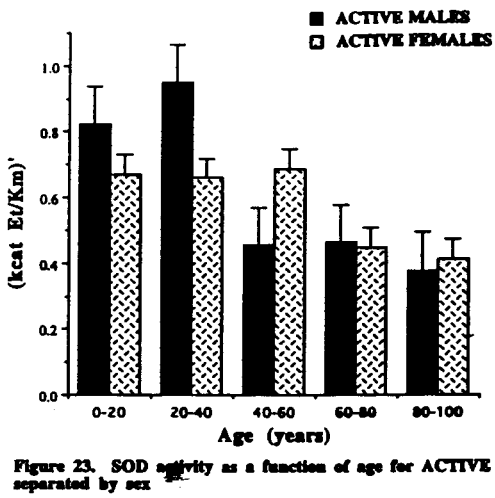

Figure 23. SOD activity as a function of age for ACTIVE subjects separated by sex

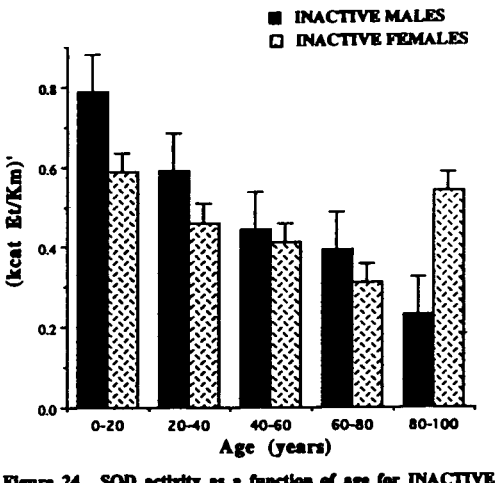

Figure 24. SOD activity as a function of age for INACTIVE subjects separated by sex

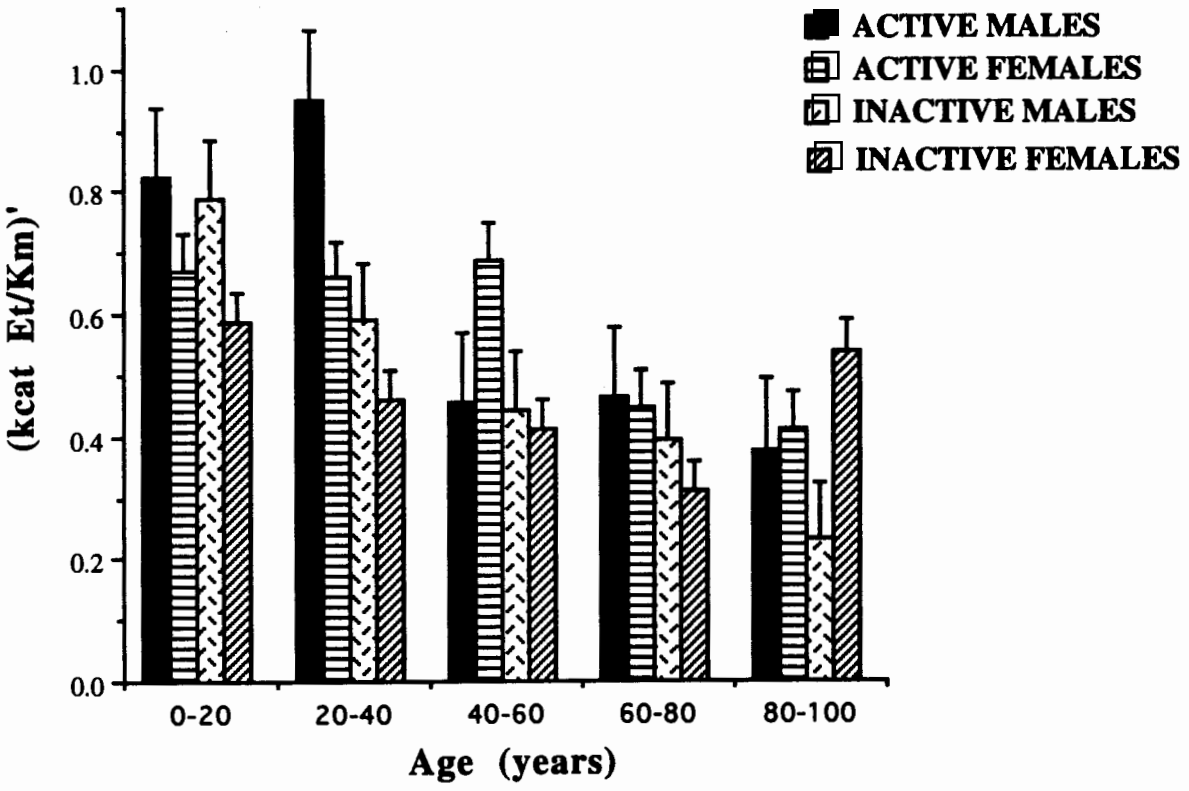

Figure 25. SOD activity as a function of age for ALL subjects separated by PHYSICAL ACTIVITY and SEX

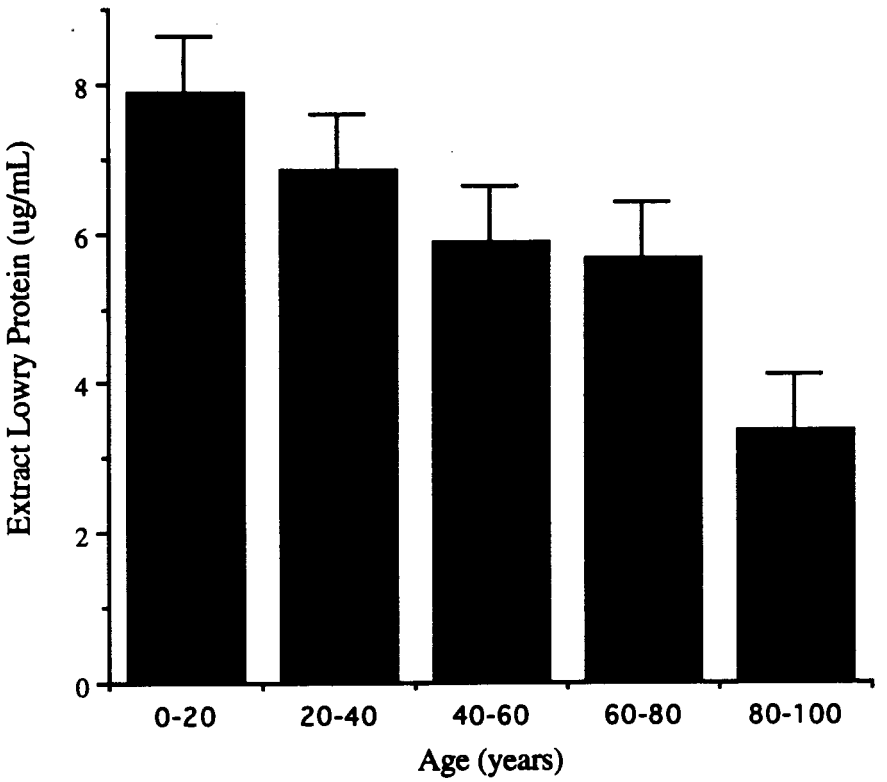

Figure 26. Total extract protein concentration as a function of age for ALL subjects

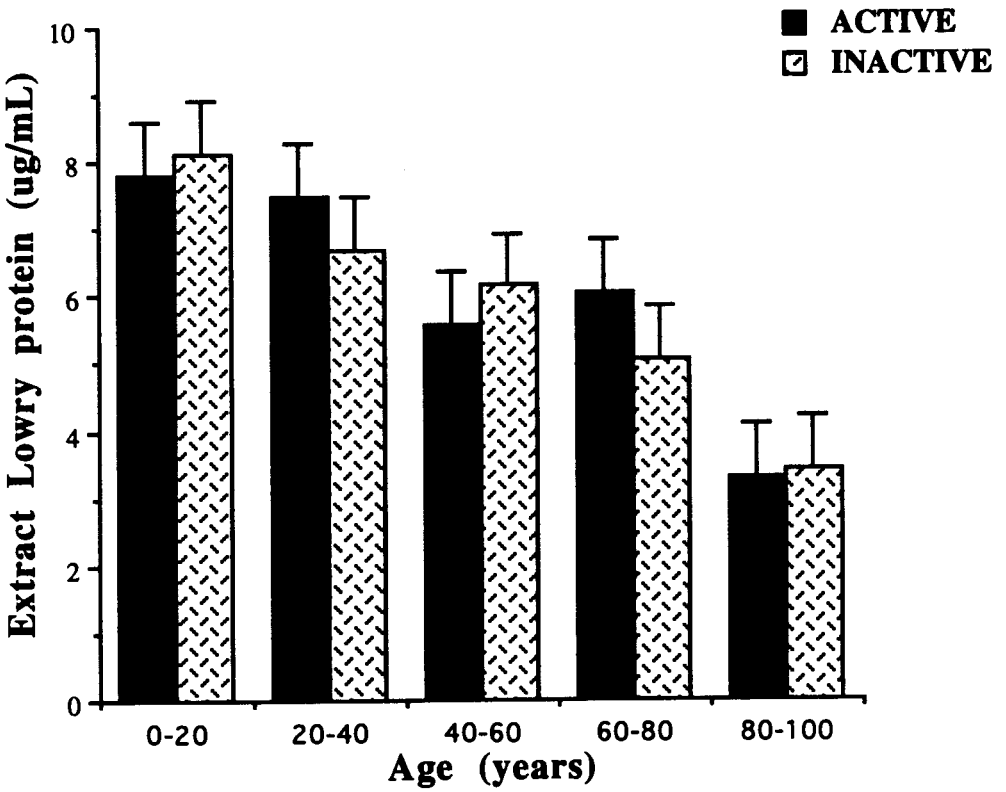

Figure 27. Total extract protein concentrations as a function of age for ALL subjects separated by PHYSICAL ACTIVITY

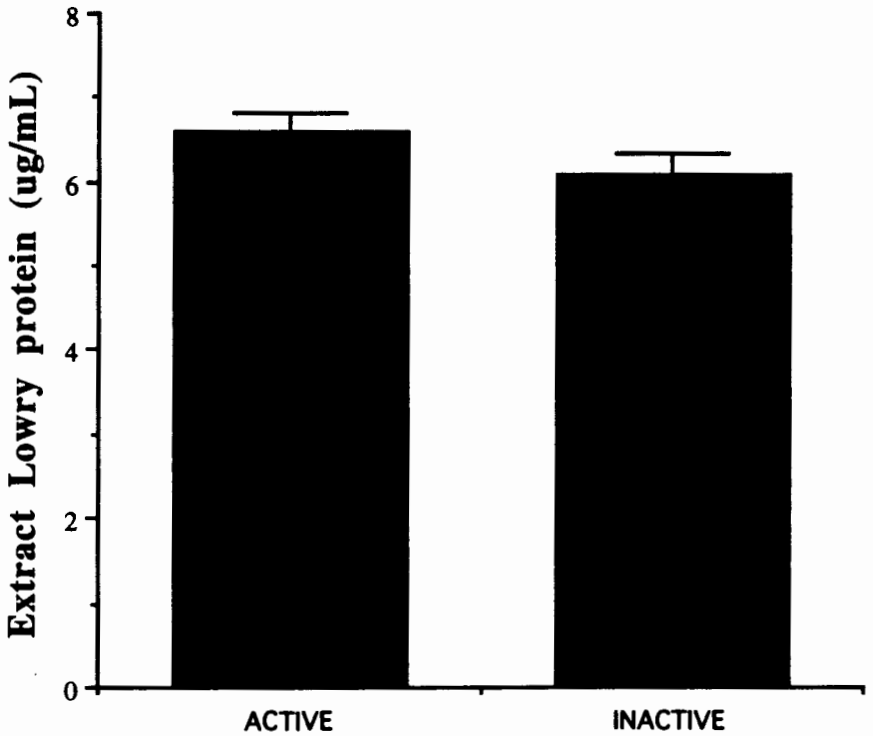

Figure 28. Mean Lowry protein concentration in extracts of ACTIVE and INACTIVE subjects

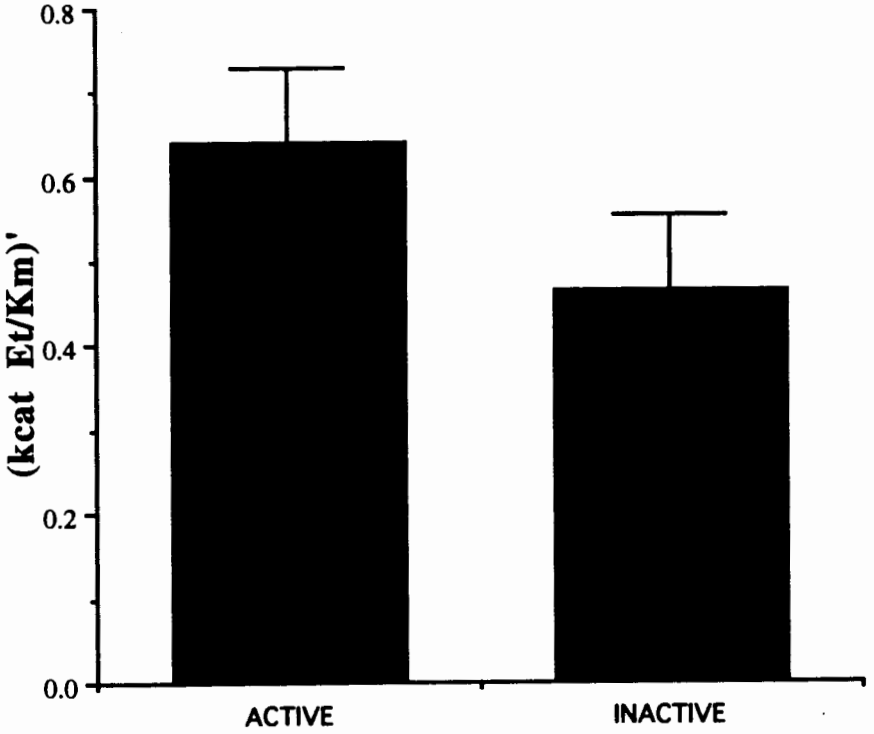

Figure 29. Mean SOD activity in extracts from ACTIVE and INACTIVE subjects

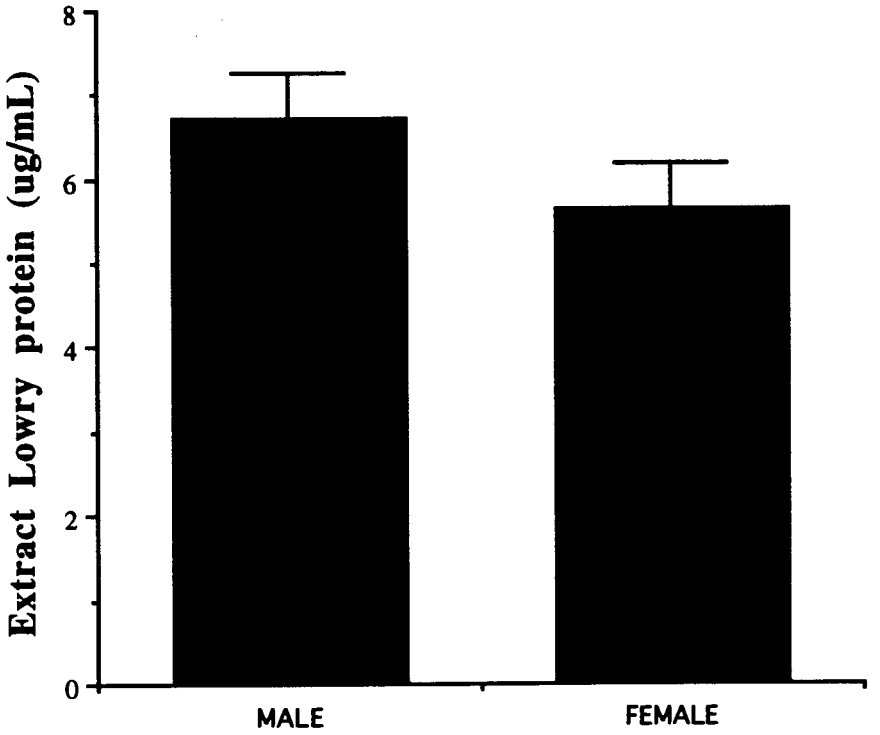

Figure 30. Mean Lowry protein concentration in extracts of MALE and FEMALE samples

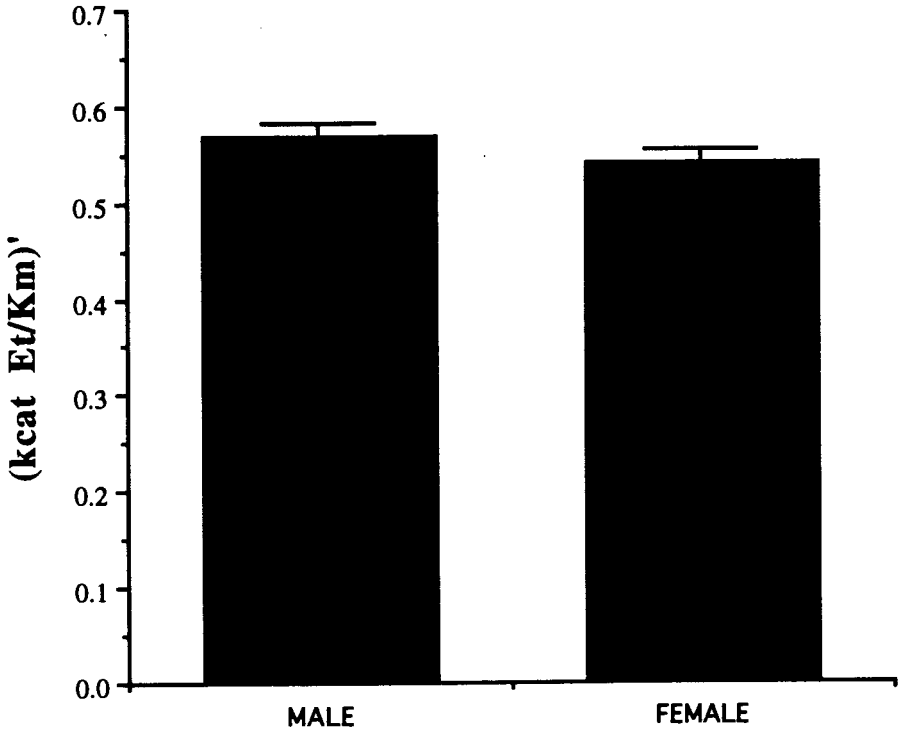

Figure 31. Mean SOD activity in extracts from MALE and FEMALE subjects

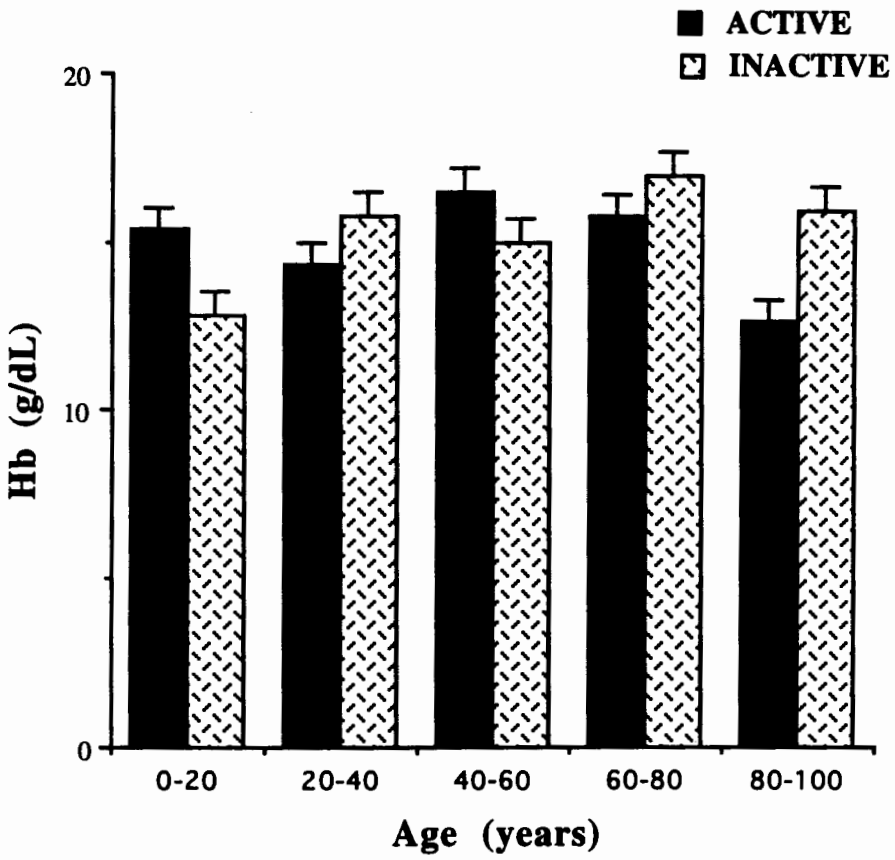

Figure 32. Drabkin hemoglobin concentrations as a function of age in ALL subjects separated by PHYSICAL ACTIVITY

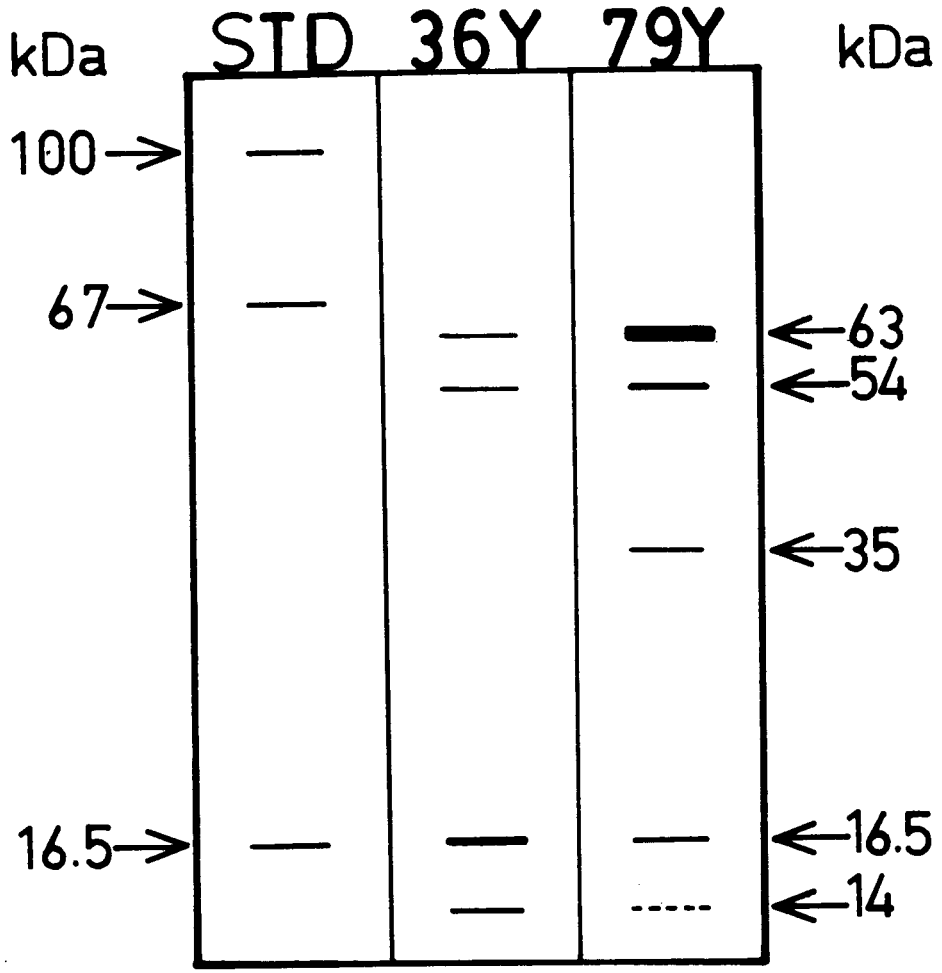

Figure 33.

GEL ELECTROPHORESIS OF EXTRACT PROTEINS

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Appendix A

Free Radicals

A free radical is a species which has one or more unpaired electron(s) (Halliwell and Gutteridge, 1985). According to this broad definition, the hydrogen atom, the oxygen molecule and most transition metals can be classified as free radicals (Halliwell and Gutteridge, 1985). Free radicals have an unpaired electron in an outer orbital and therefore they are very unstable and highly reactive molecular species which seek to either abstract an electron from a neighboring molecule or donate the unpaired electron to an electron deficient species.

When a free radical is introduced into a stable biochemical pool of molecules it can reek havoc on the cell. This is because once electron abstraction is initiated, chain reaction hydrogen abstractions are propagated which result in massive, biochemical alterations. One of the most deleterious effect of oxygen free radicals in biological systems appears to be lipid peroxidation. In particular, the abundance of allylic sites in polyunsaturated fatty acids makes them especially susceptible to lipid peroxidation (Jenkins, 1988). When such an event occurs enzyme functions may be altered. For example, succinic dehydrogenase and N-hydroxybutyrate dehydrogenase are found to be relatively inactive when phospholipids are removed from a reaction medium, but regain activity when phospholipids are replaced (Demopoulous, 1973). Membrane alteration may change an enzyme's configuration so that the active sites becomes unavailable/hindered. De Groot *et al.* (1985) found a loss of liver microsomal enzyme latent activity which was interpreted as being the result of a loss in proper orientation of the enzyme active site in the microsomal lumen. Similarly, Nohl *et al.* (1986) reported that ubiquinone (an important mitochondrial coenzyme) was affected by changes in phospholipid orientation.

Another important consequence of lipid peroxidation is the influence it has on membrane fluidity. Chia *et al.* (1983) reported that $O_2^{\cdot-}$ caused human myelin to change from a

crystalline state to a disordered liquid crystalline state. Moreover, changes in fluidity may cause membranes to become leaky with the consequent loss of intracellular enzymes and efflux of hydrolytic enzymes. It has been shown that even brief bouts of lipid peroxidation cause long lasting membrane damage (Flohe, 1978).

Also, lipid peroxidation reaction products are not restricted to the site of production, but may diffuse away causing further damage at a distance (Benedetti *et al.*, 1979). Lipid peroxides are capable of initiating lipid peroxidation at remote sites and hydrogen peroxide may haemolyse red blood cells. Aldehydes and hydroxyl alkenals react with DNA and may produce mutagenesis or carcinogenesis (Kappur, 1986).

However, it is important to note that although most studies have concentrated on the harmful effects of radicals, reactive oxygen species may play an important role in many physiological functions. The "respiratory burst" killing of pathogens by neutrophils and macrophages is the best documented of these beneficial roles (Gabig and Babior, 1981). It has even been speculated that $O_2^{\cdot-}$ may be essential to normal tissue function and that it may be reduced beyond a critical level when elevated SOD activity prevails (Michelson, 1977; Del Maestro, 1980). Furthermore, SOD has been shown to be elevated in the following disorders (although no direct link has yet been elucidated): autism, paranoia, schizophrenia, Werner's syndrome, Down's syndrome and Alzheimer's disease (Marklund, 1986).

Also, oxygen free radicals are known to be involved in several important reactions which include enzyme activation (Nagui *et al.*, 1986), sparking endoperoxidation (Nagui *et al.*, 1986; Needleman *et al.*, 1986), and drug detoxification (Kappus, 1985). Radicals may also regulate biogenesis of mitochondria or whole cells (Oberley *et al.*, 1981; Morasaki *et al.*, 1982).

Appendix B

Spin Restriction

Ground state molecular oxygen contains two unpaired electrons in parallel spin states (i.e. the same spin quantum number). Each of these two unpaired electrons is located in a different pi* antibonding orbital (Halliwell and Gutteridge, 1985). Thus, oxygen is paramagnetic (Fridovich, 1977a). Divalent reduction of oxygen in which a pair of electrons is inserted into the molecule is disallowed as it would result in a situation in which two electrons with parallel spins occupy the same orbital. This would be a violation of the Pauli Exclusion Principle which states that "in a many electron atom, no two electrons can have identical values for all four quantum numbers" (Segal, 1985). This imposes a restriction on oxidations by oxygen which tends to make oxygen accept electrons one at a time (Halliwell and Gutteridge, 1985). This spin restriction, which hinders divalent reduction and favors a univalent pathway of oxygen reduction, makes molecular oxygen much less reactive than would be otherwise expected. Also, univalent reduction makes superoxide a common intermediate of oxygen reduction.

The spin restriction can be circumvented in three ways (Fridovich, 1977a):

(1) Electronic excitation can move one of the electrons from the ground state orbital to a more energetic orbital and invert its spin state. In fact, a minimum of 23 kcal/mol can convert the triplet ground state of molecular oxygen into a very reactive singlet excited state.

(2) Oxygen can ligate to a transition metal which contains its own unpaired electronic spins. Such a complex can accept a pair of electrons without violating the need to conserve angular momentum.

(3) Electrons can be added to oxygen one at a time via a univalent reduction pathway. This pathway inevitably generates intermediates, the first being superoxide. When a single electron

is accepted by the ground-state oxygen molecule it enters one of the pi* antibonding orbitals. The univalent reduction of oxygen occurs commonly due to the spin restriction and therefore superoxide is often a by-product of oxidative reactions (Fridovich, 1977a). In aqueous solutions superoxide dismutates to hydrogen peroxide and oxygen. The rate of dismutation increases at acidic pH values (McCord *et al.*, 1977).

Thus, the stepwise reduction of oxygen to water in the mitochondrial respiratory chain, which requires four electrons, proceeds most readily by a univalent pathway which involves dangerously reactive intermediates (Fridovich, 1977a).

Transition metals are found at the active sites of many oxidase and oxygenase enzymes because they are capable of accepting and donating single electrons (Halliwell and Gutteridge, 1985).

The addition of a second electron to superoxide yields a peroxide ion (O_2^{2-}) which has no unpaired electrons (Halliwell and Gutteridge, 1985). Any peroxide formed at physiological pH (7.4) will immediately protonate to produce hydrogen peroxide as the pKa of hydrogen peroxide is quite high (Halliwell and Gutteridge, 1985). The pKa of $HO_2^{\cdot}$ in aqueous solution is 4.8 (Behar *et al.*, 1970). Homolytic fission of the relatively weak O-O bond in hydrogen peroxide (the dissociation energy is only 38 kcal/mol) gives rise to two hydroxy radicals. This homolytic cleavage can be achieved by heat or by ionizing radiation (Halliwell and Gutteridge, 1985).

Also, mixtures of hydrogen peroxide and an iron(II) salt will produce hydroxyl radicals via the Fenton reaction (Walling, 1982):

$$Fe^{+2} + H_2O_2 \text{ ---> } Fe^{+3} + {}^{\cdot}OH + OH^-$$

Traces of Fe^{+3} can also react with hydrogen peroxide (Halliwell and Gutteridge, 1985).

$$Fe^{+3} + H_2O_2 \text{ ---> } Fe^{+2} + O_2^{\cdot-} + 2H^+$$

Hence, a whole series of radical reactions can be initiated (Halliwell and Gutteridge, 1985).

i.e. $$\cdot OH + H_2O_2 \text{ ---> } H_2O + H^+ + O2^{\cdot-}$$

$$O_2^{\cdot-} + Fe^{+3} \text{ ---> } Fe^{+2} + O_2$$

$$\cdot OH + Fe^{+2} \text{ ---> } Fe^{+3} + OH^- \quad \text{etc.}$$

Appendix C

Sources of Superoxide

Many biologically important substances autoxidize and thereby generate superoxide. These include the reduced flavins, hydroquinones, catecholamines, tetrahdropterins, hemoproteins, and reduced ferrodoxins (Fridovich, 1977b). Superoxide is formed in almost all aerobic cells.

Also, many oxidative enzymes have been shown to produce considerable amounts of superoxide (Fridovich, 1977b). Such an enzyme is xanthine oxidase. The production of superoxide by subcellular organelles in the presence of an electron source and oxygen is well documented (Fridovich, 1977b). In fact, the major source of superoxide is "leakage" of electrons onto oxygen from various components of the mitochondria and as oxygen concentrations are increased, the amount of "leakage" and therefore the rate of superoxide production increases (Freeman and Crapo, 1981). Chloroplast fragments are also known to produce superoxide (Asada *et al.*, 1977).

Furthermore, there is evidence that specialized (whole) cells such as phagocytes produce superoxide and release it to their surrounding medium during respiratory burst intended to destroy foreign particles or immune complexes (antimicrobial/antibactericidal action) (Freeman and Crapo, 1982; Halliwell, 1982). This is an important *in vivo* source of oxygen free radicals. Phagocytic cells known to produce superoxide include neutrophils, monocytes, eosinophils and macrophages (Halliwell and Gutteridge, 1985). The phagocytic killing of engulfed microorganisms is dependent on superoxide production by a membrane bound NADPH oxidase (also known as a superoxide synthetase) during oxygen reduction. It has been postulated that the K_m for oxygen of the NADPH complex is within the range of oxygen concentrations in body fluids (Halliwell, 1982). Thus, an elevation in oxygen

concentration may increase superoxide production by activated phagocytes (Edwards *et al.*, 1983).

Moreover, a considerable amount of superoxide generated within cells comes from membrane-bound systems (Fridovich, 1974; Fridovich, 1975; Fridovich, 1978) and probably some superoxide is formed in the membrane interior as oxygen is 7-8 fold more soluble in nonpolar (organic) solvents than it is in water (aqueous solvents) (Subszynski and Hyde, 1983). Hence, there is somewhat of a "partitioning" of oxygen into the interior of membranes which are known to be hydrophobic. Ionizing radiation is another source of superoxide in aerated aqueous systems.

It should be noted that superoxide generated outside a cell does not penetrate the membrane (Takahashi and Asada, 1983). An exception to this is the erythrocyte membrane which contains an "anion channel" through which superoxide can pass (Lynch and Fridovich, 1978). Hydrogen peroxide readily crosses membranes to enter cells thereby increasing intracellular production of hydroxyl radicals (Melho-Filho *et al.*, 1984). Externally generated hydroxyl radicals react immediately with the first membrane component that they encounter and therefore hydroxyl radicals never cross membranes (Halliwell and Gutteridge, 1985). The following is a list of intracellular sources of superoxide (Frank, 1985):

Intracellular Sources of Superoxide

Enzymes

Xanthine oxidase

Aldehyde oxidase

Tryptophan dioxygenase

Indolamine dioxygenase

Flavoprotein dehydrogenase

Autoxidation

Reduced ---> oxidized ferrodoxin

Adrenaline ---> adrenochrome

Hemoglobin ---> methemoglobin

Certain flavins, thiols, hydroquinones, etc.

Mitochondria

Ubiquinone-cytochrome b portion of the respiratory chain

Microsomes

"Uncoupled" cytochrome P-450 reactions

Appendix D

Cytotoxic Oxygen Species

$O_2^{\cdot-}$	Superoxide radical
H_2O_2	Hydrogen Peroxide
$^{\cdot}OH$	Hydroxyl radical
$^1O_2$	Singlet oxygen
$ROO^{\cdot}$	Peroxide radical (R = lipid)

Appendix E

Antioxidant Defenses

Enzymatic

Superoxide dismutase (SOD)

Catalase (CAT)

Glutathione Peroxidase (GPX) +

Glutathione Reductase (GR) and

Glucose-6-phosphate dehydrogenase (G-6-PD)

Nonenzymatic

Vitamin E

beta-Carotene

Ascorbate

Glutathione

Also: cysteamine, cysteine, nonessential PUFA, thiols

Appendix F

Cell Components Damaged by Reactive Oxygen Species

Lipids -- Peroxidation of unsaturated fatty acids in organelle plasma membranes

Proteins -- Oxidation of sulfhydryl-containing enzymes --> enzyme inactivation

Carbohydrates -- Polysaccharide depolymerization

Nucleic Acids -- Base hydroxylations, "nicking", cross-linkage, scission of DNA strands (mutations)

Also, inhibitory effects on protein, nucleotide and fatty acid biosynthesis.

Appendix G

Harmful Consequences of Radical-Mediated Chemistry

Aging

Cancer

Cataracts

Environmental nitrogen dioxide - ozone

Cytostatic damage - doxorubicin (adriamycin)

Hyperbaric O_2 toxicity

Radiation injury

Postischaemic damage

Keshan's disease

Diabetes

Appendix H

Evidence for the Free Radical Theory of Aging:

Support for the Free Radical Theory of Aging comes from many realms. Researchers testing this theory have investigated alterations in antioxidant levels with age, alterations in prooxidant production with age, and the effect of metabolic rate on free radical production and longevity.

The hypothesis that free radical-induced damage mediates aging is strengthened by findings which indicate that the rate of lipid peroxidation is directly proportional to ambient temperature in several poikilothermic species (Sohal *et al.*, 1981; McArthur and Sohal, 1982; Sohal *et al.*, 1985; Sestini and Allsopp, 1991). Sohal *et al.* (1985) have reported that with an increase in ambient temperature there is a corresponding increase in metabolic rate of *Drosophila* flies which also correlates with a decrease in longevity. In addition, Miquel *et al.* (1983) and Fleming *et al.* (1987) have also noted a negative correlation between metabolic rate and lifespan in different strains of wildtype *Drosophila* flies. McArthur and Sohal (1982) using another experimental model, the milkweed bug, also observed declines in average and maximum lifespans of these bugs due to ambient temperature accelerated metabolic rates. Furthermore, Sestini and Allsopp (1991) report that elevated temperatures, which lead to an increase in metabolic activity and respiration in insects, accelerated the production of free radicals. Consequently, greater cell damage and reduced longevity was observed. Sestini and Allsopp's data support several other studies which have demonstrated a strong correlation between metabolic rate, oxygen consumption, physical activity and lifespan in poikilotherms. These correlations between metabolic rate and lifespan have been attributed to the deleterious effects of free radical reactions which are presumed to be responsible for loss of cell functions (Fridovich, 1976; Farmer and Sohal, 1987). Thus, there is considerable evidence suggesting

that an increase in ambient temperature, by accelerating the production of free radicals will hasten the degenerative processes associated with aging and will ultimately shorten the organism's lifespan (Poot, 1991).

Since superoxide dismutase (SOD) is believed to be one of the most important protective enzymes for removing the reactive by-products of oxygen metabolism (Jozwiak and Josnowska, 1985; Somville *et al.*, 1985), several researchers have concentrated on elucidating the relationship between metabolic rate, SOD activity and lifespan. Tolmasoff *et al.* (1980) found the ratio of metabolic rate to SOD activity to be related to lifespan in primate species. Furthermore, Del Maestro and McDonald (1987) investigating within animal variations in SOD, noted that the aging rate of different rat tissues correlated with SOD activity. Similar evidence has prompted Lopez-Torres *et al.* (1991) to suggest that the variations in the rates of physiological scavenging of free radicals by defensive antioxidant systems during an organism's lifespan may lead to different rates of aging at different ages. Mailer *et al.* (1991) probed the link between antioxidative enzyme activity and lifespan by comparing normal hearts to almost identical ones under cardiomyopathic stress. They found that membrane abnormalities and a shortened lifespan were closely associated with the progressive cardiomyopathy of dystrophic hamsters. The membrane damage appeared to be primarily due to a disruption of antioxidative defenses in the cardiomyopathic hamsters. Jozwiak and Josnowska (1985) have gone so far as proposing that a determination of superoxide dismutase, catalase and glutathione peroxidase may constitute a useful index of maturation and aging in organisms.

However, despite the general agreement that deterioration of membrane structures by free radical and peroxide mediated processes is an important part of senescence, the extent to which age-related changes in antioxidant enzymes are responsible for longevity still remains

unclear (Ischiropoulos, 1990). This is mainly because no consistent change in these enzymes has been demonstrated with respect to aging of organisms. For example, age-related increases, decreases and no change in superoxide dismutase activity have been reported (refer to section on changes in SOD with age).

The role of antioxidants and antioxidative enzymes in longevity has been approached from another angle as well: observing the effects on longevity of supplementing animals with such compounds. Several researchers have found beneficial effects, in terms of lengthened lifespans, from administering antioxidants to test animals. These experiments in which cellular antioxidant defenses have been manipulated lend support to the Free Radical Theory of Aging. For instance, administration of vitamin E and several other antioxidants has increased longevity in a number of test animals (Harman, 1961; Comfort *et al.*, 1971; Kohn, 1971; Enseco and Verdore, 1980). Abe *et al.* (1989) have shown that feeding mice *Streptococcus lactis* (SL), a lactobacillus which exists in milk and dairy products, inhibits some of the senescent deteriorations usually seen in the experimental animals. For example, Cu,Zn-SOD and Mn-SOD activities in the liver and brain of SL-fed mice were maintained at juvenile levels; whereas, the activity of these enzymes were found to decrease significantly in the commercial diet group. SL is reported to have SOD-like activity and protects against radical derived injuries. Other studies have shown no benefit from antioxidant or antioxidative enzyme supplementation.

Sohal (1991) champions the view that an increase in free radical production, rather than a decrease in antioxidant defenses, mediates rate of aging. His studies focus on an evaluation of prooxidant generation in aging insects. In a recent study, Sohal used two approaches to distinguish between the chronological age and physiological age (or life expectancy) of *Drosophila* flies. One method was to experimentally alter the lifespans of flies by

manipulating physical activity levels (a decrease in physical activity has been shown to be correlated with an increase in longevity by Sohal). His second method involved phenotypically (based on flying ability; e.g., crawlers are short-lived flies) selecting from a cohort population, subpopulations with relatively short or long lifespans. Sohal observed that the rate of mitochondrial hydrogen peroxide release correlated with the physiological age of the flies and not their chronological age. The mitochondria of flies with a shorter life expectancy (i.e., physiologically older flies) exhibited significantly accelerated rates of hydrogen peroxide production as compared to their counterparts with a longer life expectancy. Hence, Sohal claims that hydrogen peroxide production by mitochondria may be a biomarker of aging. This study supports earlier work which has shown that rates of both superoxide and hydrogen peroxide production increase with advancing age of insects (Farmer and Sohal, 1989; Sohal and Sohal, 1991) and in mammals (Sohal *et al.*, 1990).

These findings stand in direct opposition to the views of many others, including Richard Cutler, who claim that antioxidant levels are longevity determinants. Cutler, a strong proponent of the Free Radical Theory of Aging has proposed the existence of longevity determinant genes (or gerogenes) whose function is the control of life maintenance processes. One vital life maintenance process is undoubtedly the protection against oxygen free radicals provided by the body's antioxidant defense system. Moreover, an accumulation of damage with age will inevitably result in further disturbances to some essential life processes which in turn alter proper cell function (Jozwiak and Jasnowska, 1985). The loss of a critical number of cells or cell functions could conceivably lead to organ failure and ultimately organismal death. Cutler believes that all organisms possess the same basic life maintenance processes; however, genetically determined differences in efficiency and maintenance of these processes lead to the species specific differences in lifespan. In fact, the first indication that

genes may determine the rate of aging was the realization that related species have characteristically different mean lifespans.

Cutler (1986) believes that aging is an active process, which may be separate and distinct from those processes involved in general health maintenance. In his quest to understand the biological basis of human longevity he has proposed and developed the longevity determinant gene hypothesis. Some key observations which underlie this hypothesis are the following: (1) Aging is a ubiquitous process, apparently affecting every aspect of the body, (2) Scientific progress in understanding the highly complex biological aspects of aging is slow, particularly since our understanding of the normal biological nature of organisms is incomplete, (3) Mammalian species living in their natural ecological niches exhibit a wide range of lifespans, but seldom live long enough to suffer senescence. Such data suggests that aging is pleiotropic and not the result of a genetic program in which specific genes actively bring about aging. Instead, aging appears to result from the side effects of normal biological processes which are essential for maintenance of life. The differential rate of aging seen among mammalian species is likely due to how effectively these pleiotropic side effects are dealt with, presumably by longevity determinant genes, (4) Based on the observation that the molecular genetics of speciation among primates operates at the regulatory gene level, as well as other comparative and evolutionary data, differential aging rates among primates may also be due to small genetic differences governing the expression of regulatory genes, (6) In most mammalian species, aging rate is proportional to their developmental and metabolic rates. Thus, if the side effects of normal biological processes essential for life are responsible for aging, than these correlations suggest that the side effects of development and metabolism cause aging. The possible side effects that energy metabolism may have in causing aging has been extensively studied. Metabolic rate is proportional to oxygen utilization rate in aerobic

organisms. Thus, by-products of oxidative metabolism may play a pivotal role in aging. Antioxidants, the body's defenses against the toxic effects of oxygen radicals may therefore be important longevity determinants, (7) An important primary aging process in mammals may be the movement of cells away from their proper state of differentiation. This dysdifferentiation hypothesis of aging has gained support from molecular genetic studies which indicate age-dependent changes in gene expression indicative of a relaxation of proper gene regulation, (8) Even extremely low concentrations of reactive oxygen species have been shown to perturb the proper state of differentiation of cells. Administration of antioxidants, however, protects against these effects. Thus, these by-products of normal metabolic reactions may have long-term gene-destabilizing effects and antioxidants may be an important class of agents which protect against them.

Maximum lifespan potential (MLSP) is used to estimate the mean physiological aging rate of mammalian species. Although mean life span of humans has varied considerably throughout history, maximum lifespan has remained remarkably constant at around 100 years. This is also true for several other species. Thus, survival data suggests that MLSP reflects the genetic or innate biological anti-aging characteristics of species. Survival and physiological aging studies on mice, primates and humans indicate that MLSP is inversely proportional to physiological aging rate.

Cutler (1986) reports a positive correlation of antioxidant concentration per specific metabolic rate (SMR) with MLSP. The ratio of antioxidant concentration to SMR is an index of the protection of a tissue against reactive oxygen species as compared to rate of reactive oxygen species production in a given tissue. Cutler advises that one keep in mind that a linear correlation between antioxidant concentration per SMR and MLSP may not necessarily exist. Such an assumption erroneously presumes that the efficiency of production of reactive

oxygen species via oxidative metabolism is similar in all species. Instead, different innate production rates of free radicals or other toxic by-products of metabolism per unit amount of oxygen used may exist.

Also, for several mammalian species, the product of MLSP and SMR is a constant (Cutler, 1986). This has led to the definition of a new parameter of longevity termed lifespan energy potential (LEP). LEP = 2.7 MLSP x SMR, where the units of MLSP are years and the units of SMR are cal/g/day.

Since MLSP is proportional to antioxidant concentration per SMR, a new prediction emerges. Species' LEP is proportional to the total effective antioxidant concentration in animal tissues. LEP is approximately 200-300 kcal/g for most mammalian species. It is twice as great at approximately 400-660 kcal/g for most primates. The outstanding primate exceptions are human and capuchin (a South American monkey) which have a LEP in the range of 800-900 kcal/g. From these observations it appears that mammalian species differ not only in their longevity capacities with respect to absolute time (MLSP), but also they differ in the total amount of energy they are capable of using over their MLSP. Exceptionally long-lived animals, such as humans, have an unusually high MLSP (i.e., a high longevity capacity) and a high LEP (i.e., a high metabolic energy capacity).

Cutler and others have identified potential longevity determinants as those agents which exhibit either a significantly positive or negative correlation in their concentration with MLSP, LEP, or its concentration per SMR with MLSP. Many enzymes and other cellular constituents which are believed to be poor candidates as potential longevity determinants have been tested to elucidate any correlations of their tissue concentrations with MLSP or LEP. Such studies have shown no significant correlations. For example, control studies by Ono and Okada (1984) found no significant correlation between concentrations of lactate dehydrogenase,

glucose-6-phosphate dehydrogenase, glutamic-oxalacetic transaminase, creatine phosphokinase and cholinesterase in nonprimate species with MLSP. Thus, few biochemical constituents of mammalian species are significantly correlated with MLSP or LEP. Hence, significant correlations with MLSP or LEP appear to the exception, rather than the rule.

On the other hand, concentrations of good longevity determinant candidates have been shown to correlate significantly with MLSP and LEP. A prime example is SOD. Both tissue concentrations of Cu,Zn-SOD and Mn-SOD, measured in the brain, liver, and heart of primates and nonprimates, show a positive linear correlation ($r=0.727$, $P<=0.01$, $n=13$) with MLSP (Cutler, 1986). Furthermore, when the correlation between SOD per SMR versus MLSP was calculated an even higher linear correlation was observed ($r=0.961$, $P<=0.001$, $n=13$) (Cutler, 1986).

When Ono and Okada's data for nonprimate species is similarly analyzed, it is found that a positive correlation ($r=0.698$, $P<=0.02$, $n=11$) exists between SOD and MLSP (Cutler, 1986). When SOD concentration per SMR is plotted against MLSP na even higher correlation is obtained ($r=0.825$, $P<=0.001$, $n=11$) (Cutler, 1986).

These data essentially show that LEP of a species is proportional to tissue SOD concentrations and suggest that LEP may be determined (at least in part) by the net antioxidant efficiency of an organism's cells (Cutler, 1986). Several other antioxidants have also been examined and can be summarized as follows (Cutler, 1986):

(1) SIGNIFICANT POSITIVE CORRELATION OF MLSP VERSUS ANTIOXIDANT CONCENTRATION PER SMR (humans have highest concentration)

SOD (Cu,Zn and Mn types)
Urate
Carotenoids
Alpha-tocopherol
Ceruloplasmin
Ascorbate (adult brain)

(2) NO SIGNIFICANT CORRELATION OF MLSP VERSUS ANTIOXIDANT CONCENTRATION PER SMR (humans no different than short-lived species)

Ascorbate
Retinol

(3) SIGNIFICANT NEGATIVE CORRELATION OF MLSP VERSUS ANTIOXIDANT CONCENTRATION PER SMR (humans have lowest concentration)

Catalase
Glutathione
Glutathione peroxidase

*Note: These results are contrary to what would be expected and can be viewed as strong evidence against the importance of reactive oxygen species and detoxification processes in aging.

(4) SIGNIFICANT NEGATIVE CORRELATION OF MLSP VERSUS DETOXIFICATION CAPACITY (humans have lowest concentration)

Cytochrome P-450 (liver)
Glutathione Transferase (liver)

(5) SIGNIFICANT POSITIVE CORRELATION OF MLSP VERSUS RESISTANCE OF TISSUES TO AUTOXIDATION (humans have highest resistance)

Important contributing factors: antioxidants, tissue structure and tissue lipid composition. uncoupled cyt p-450 reactions

A number of strategies appear to be effective in reducing the possible aging effects of reactive oxygen species. These include: an absolute increase of antioxidant concentration, a decrease in the amount of tissue peroxidizable material, a decrease in SMR, and a decrease in the extent to which toxic metabolic reactions exist in tissues (Cutler, 1986). Cutler notes that these putative strategies all involve a quantitative change in gene expression rather than a qualitative one.

Sohal (1991) argues that Cutler's hypothesis that antioxidant levels are longevity determinants is flawed due to four main reasons: (1) High levels of SOD and low levels of glutathione peroxidase and catalase would increase intracellular hydrogen peroxide concentration and hence hydroxyl radical generation, (2) Overexpression of SOD in genetic transformants has been shown to increase oxidative stress in *E. coli* and does not prolong lifespan of *Drosophila* flies, (3) Lab studies have not conclusively shown any significant declines in overall levels of antioxidant defenses with age in several insects and mammals, and (4) Administration of low molecular weight antioxidants does not alter maximum lifespan. Based on these considerations, Sohal claims that alterations in antioxidant defenses cannot account for either individual aging or variations in MLSP of different species.

Nonetheless, a review of the literature indicates that although the Free Radical Theory of Aging is not yet uniformally accepted, strong evidence exists suggesting that aging is mediated by the deleterious and irreversible changes in the body brought about by free radical reactions (Ceballos-Picot *et al.*, 1992). Great controversy still prevails; however, as to whether aging is associated with increases, decreases or no change in cellular antioxidant functions. Thus, much more research is necessary to resolve the role of reactive oxygen species, antioxidants, and detoxification reactions in mediating the aging process (Cutler, 1986). Many studies support to the hypothesis that SOD activity levels determine or at least are correlated

with the rate of aging.

Appendix I

QUESTIONNAIRE OF CURRENT PHYSICAL ACTIVITY IN THE PREVIOUS YEAR

1.) Number of stairs ascended daily

2.) Number of city blocks walked daily

3.) Sports played Time per event Days per week

Light sports only

Vigorous sports with light sports

Vigorous sports only

4.) Specify the sports played

5.) Has there been a recent change in activity level? If yes, when?

6.) Sex

7.) Birthdate

8.) Height

9.) Weight

10.) Any medications? If yes, which ones?

11.) Do you smoke?

This questionnaire was scored via the method of Paffenbarger *et al.* (1983) in order to compute a physical activity index in kcal/wk. Sports were classified as light (5 kcal/wk), mixed (7.5 kcal/wk), and vigorous (10 kcal/wk). It is important to note that the physical activity index is an indicator rather than an absolute measure of energy expenditure. The following scoring scheme was used.

SCORING

1.) 20 stairs = 2 flights or one story = 8 kcal

2.) walking one city block = $1/12^{th}$ mile = 8 kcal

3.) Light sports = 5 kcal/min

(Light sports include bowling, baseball, golf, yard work, etc.)

4.) Vigorous sports = 10 kcal/min

(Vigorous sports include running, swimming, cross country skiing, etc.)

5.) Combination light and vigorous sports = 7.5 kcal/min

CALCULATION OF KILOCALORIES PER WEEK

1.) Number of flights per day * 8 kcal/flight * 7 days/wk

2.) Number of city blocks per day * 8 kcal/city block * 7 days/wk

3.) Time spent playing light sports per day * 5 kcal/min * number of days played/week

4.) Time spent playing vigorous sports per day * 10 kcal/min * number of days played/week

5.) Time spent playing combination (light & vigorous) sports per day * 7.5 kcal/min * number of days played/week

Total energy expenditure per week equals summation of 1-5 above.

Subjects were classified as ACTIVE if their physical activity index was greater than 1500 kcal/wk or INACTIVE if their physical activity index was less than 1500 kcal/wk.

Appendix J

Analysis of Variance Procedure
Class Level Information

Class	Levels	Values
GROUP	10	0 1 2 3 4 5 6 7 8 9
AI	2	0 1
MF	2	0 1
AGE20	5	0 1 2 3 4

Number of observations in data set = 130

Group	Obs	Dependent Variables
1	96	INVSL
2	127	AGE PA
3	115	HB
4	112	L
5	101	B
6	80	TEMP LNTEMP

NOTE: Variables in each group are consistent with respect to the presence or absence of missing values.

Analysis of Variance Procedure

Dependent Variable: INVSL

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	2.42912611	0.26990290	6.91	0.0001
Error	86	3.35769032	0.03904291		
Corrected Total	95	5.78681643			

R-Square	C.V.	Root MSE

INVSL Mean

0.419769 35.34995 0.19759279
0.55896207

Source	DF	Anova SS	Mean Square	F Value	Pr > F
GROUP	9	2.42912611	0.26990290	6.91	0.0001

Analysis of Variance Procedure

T tests (LSD) for variable: INVSL

NOTE: This test controls the type I comparisonwise error rat
e not the
experimentwise error rate.

Alpha= 0.05 Confidence= 0.95 df= 86 MSE= 0.039043
Critical Value of T= 1.98793

Comparisons significant at the 0.05 level are indicated by '***

GROUP Comparison	Lower Confidence Limit	Difference Between Means	Upper Confidence Limit	
2 - 0	-0.13676	0.01325	0.16325	
2 - 1	-0.00130	0.19995	0.40120	
2 - 3	0.09045	0.22933	0.36821	***
2 - 4	0.14400	0.29400	0.44400	***
2 - 6	0.16643	0.33010	0.49377	***
2 - 5	0.20515	0.36349	0.52184	***
2 - 8	0.09662	0.39123	0.68583	***
2 - 7	0.24842	0.41209	0.57576	***
2 - 9	0.29210	0.49335	0.69460	***
0 - 2	-0.16325	-0.01325	0.13676	
0 - 1	-0.02238	0.18670	0.39579	
0 - 3	0.06608	0.21608	0.36609	***
0 - 4	0.12039	0.28075	0.44111	***
0 - 6	0.14364	0.31685	0.49006	***
0 - 5	0.18206	0.35025	0.51844	***
0 - 8	0.07797	0.37798	0.67799	***
0 - 7	0.22563	0.39884	0.57205	***
0 - 9	0.27102	0.48010	0.68919	***
1 - 2	-0.40120	-0.19995	0.00130	
1 - 0	-0.39579	-0.18670	0.02238	

1	- 3	-0.17187	0.02938	0.23063	
1	- 4	-0.11503	0.09405	0.30314	
1	- 6	-0.08894	0.13015	0.34925	
1	- 5	-0.05160	0.16355	0.37869	
1	- 8	-0.13736	0.19128	0.51992	
1	- 7	-0.00695	0.21214	0.43123	
1	- 9	0.04497	0.29340	0.54183	***
3	- 2	-0.36821	-0.22933	-0.09045	***
3	- 0	-0.36609	-0.21608	-0.06608	***
3	- 1	-0.23063	-0.02938	0.17187	
3	- 4	-0.08533	0.06467	0.21467	
3	- 6	-0.06290	0.10077	0.26444	
3	- 5	-0.02418	0.13416	0.29251	
3	- 8	-0.13271	0.16190	0.45650	
3	- 7	0.01909	0.18276	0.34643	***
3	- 9	0.06277	0.26402	0.46527	***
4	- 2	-0.44400	-0.29400	-0.14400	***
4	- 0	-0.44111	-0.28075	-0.12039	***
4	- 1	-0.30314	-0.09405	0.11503	
4	- 3	-0.21467	-0.06467	0.08533	
4	- 6	-0.13711	0.03610	0.20931	
4	- 5	-0.09869	0.06949	0.23768	
4	- 8	-0.20278	0.09723	0.39723	
4	- 7	-0.05512	0.11809	0.29130	
4	- 9	-0.00974	0.19935	0.40843	
6	- 2	-0.49377	-0.33010	-0.16643	***
6	- 0	-0.49006	-0.31685	-0.14364	***
6	- 1	-0.34925	-0.13015	0.08894	
6	- 3	-0.26444	-0.10077	0.06290	
6	- 4	-0.20931	-0.03610	0.13711	
6	- 5	-0.14709	0.03339	0.21387	
6	- 8	-0.24594	0.06113	0.36819	
6	- 7	-0.10318	0.08199	0.26716	
6	- 9	-0.05585	0.16325	0.38234	
5	- 2	-0.52184	-0.36349	-0.20515	***
5	- 0	-0.51844	-0.35025	-0.18206	***
5	- 1	-0.37869	-0.16355	0.05160	
5	- 3	-0.29251	-0.13416	0.02418	
5	- 4	-0.23768	-0.06949	0.09869	
5	- 6	-0.21387	-0.03339	0.14709	
5	- 8	-0.27653	0.02773	0.33199	
5	- 7	-0.13188	0.04859	0.22907	
5	- 9	-0.08529	0.12985	0.34500	
8	- 2	-0.68583	-0.39123	-0.09662	***
8	- 0	-0.67799	-0.37798	-0.07797	***
8	- 1	-0.51992	-0.19128	0.13736	
8	- 3	-0.45650	-0.16190	0.13271	
8	- 4	-0.39723	-0.09723	0.20278	
8	- 6	-0.36819	-0.06113	0.24594	
8	- 5	-0.33199	-0.02773	0.27653	
8	- 7	-0.28620	0.02086	0.32793	
8	- 9	-0.22652	0.10212	0.43076	

7	- 2	-0.57576	-0.41209	-0.24842 ***

Analysis of Variance Procedure

GROUP Comparison		Lower Confidence Limit	Difference Between Means	Upper Confidence Limit	
7	- 0	-0.57205	-0.39884	-0.22563	***
7	- 1	-0.43123	-0.21214	0.00695	
7	- 3	-0.34643	-0.18276	-0.01909	***
7	- 4	-0.29130	-0.11809	0.05512	
7	- 6	-0.26716	-0.08199	0.10318	
7	- 5	-0.22907	-0.04859	0.13188	
7	- 8	-0.32793	-0.02086	0.28620	
7	- 9	-0.13784	0.08126	0.30035	
9	- 2	-0.69460	-0.49335	-0.29210	***
9	- 0	-0.68919	-0.48010	-0.27102	***
9	- 1	-0.54183	-0.29340	-0.04497	***
9	- 3	-0.46527	-0.26402	-0.06277	***
9	- 4	-0.40843	-0.19935	0.00974	
9	- 6	-0.38234	-0.16325	0.05585	
9	- 5	-0.34500	-0.12985	0.08529	
9	- 8	-0.43076	-0.10212	0.22652	
9	- 7	-0.30035	-0.08126	0.13784	

Analysis of Variance Procedure

Dependent Variable: AGE

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	51310.19943191	5701.13327021	192.79	0.0001
Error	117	3459.81779158	29.57109224		
Corrected Total	126	54770.01722350			

R-Square	C.V.	Root MSE	AGE Mean
0.936830	12.48949	5.43793088	43.54006299

Source	DF	Anova SS	Mean Square	F Value	Pr > F

GROUP		9	51310.19943191	5701.13327021
192.79	0.0001			

Analysis of Variance Procedure

Dependent Variable: PA

Source	DF	Sum of Squares	Mean Square
F Value Pr > F			
Model	9	621563736.61013100	69062637.40112570
7.97 0.0001			
Error	117	1013847122.58215000	8665360.02206967
Corrected Total	126	1635410859.19228000	

R-Square	C.V.	Root MSE	PA Mean
0.380066	103.0053	2943.69835786	2857.81181102

Source	DF	Anova SS	Mean Square
F Value Pr > F			
GROUP	9	621563736.61013100	69062637.40112570
7.97 0.0001			

Analysis of Variance Procedure

T tests (LSD) for variable: AGE

NOTE: This test controls the type I comparisonwise error rat
e not the
experimentwise error rate.

Alpha= 0.05 Confidence= 0.95 df= 117 MSE= 29.57109
Critical Value of T= 1.98045

Comparisons significant at the 0.05 level are indicated by '***

GROUP Comparison	Lower Confidence Limit	Difference Between Means	Upper Confidence Limit
9 - 8	-7.592	1.418	10.428

9	- 6	8.621	14.232	19.843	***
9	- 7	10.623	16.522	22.421	***
9	- 4	28.818	34.336	39.854	***
9	- 5	30.024	35.469	40.913	***
9	- 3	43.028	48.413	53.797	***
9	- 2	47.963	53.348	58.733	***
9	- 0	63.880	69.359	74.838	***
9	- 1	64.901	71.712	78.523	***
8	- 9	-10.428	-1.418	7.592	
8	- 6	4.673	12.814	20.955	***
8	- 7	6.762	15.104	23.446	***
8	- 4	24.841	32.918	40.995	***
8	- 5	26.023	34.051	42.078	***
8	- 3	39.008	46.995	54.981	***
8	- 2	43.943	51.930	59.917	***
8	- 0	59.891	67.941	75.992	***
8	- 1	61.284	70.294	79.304	***
6	- 9	-19.843	-14.232	-8.621	***
6	- 8	-20.955	-12.814	-4.673	***
6	- 7	-2.169	2.290	6.749	
6	- 4	16.163	20.104	24.045	***
6	- 5	17.399	21.236	25.074	***
6	- 3	30.427	34.180	37.933	***
6	- 2	35.363	39.116	42.869	***
6	- 0	51.240	55.127	59.014	***
6	- 1	51.869	57.480	63.091	***
7	- 9	-22.421	-16.522	-10.623	***
7	- 8	-23.446	-15.104	-6.762	***
7	- 6	-6.749	-2.290	2.169	
7	- 4	13.473	17.814	22.155	***
7	- 5	14.699	18.947	23.194	***
7	- 3	27.719	31.891	36.062	***
7	- 2	32.655	36.826	40.997	***
7	- 0	48.545	52.837	57.129	***
7	- 1	49.291	55.190	61.089	***
4	- 9	-39.854	-34.336	-28.818	***
4	- 8	-40.995	-32.918	-24.841	***
4	- 6	-24.045	-20.104	-16.163	***
4	- 7	-22.155	-17.814	-13.473	***
4	- 5	-2.568	1.132	4.833	
4	- 3	10.464	14.076	17.689	***
4	- 2	15.400	19.012	22.624	***
4	- 0	31.272	35.023	38.774	***
4	- 1	31.858	37.376	42.894	***
5	- 9	-40.913	-35.469	-30.024	***
5	- 8	-42.078	-34.051	-26.023	***
5	- 6	-25.074	-21.236	-17.399	***
5	- 7	-23.194	-18.947	-14.699	***
5	- 4	-4.833	-1.132	2.568	
5	- 3	9.445	12.944	16.443	***
5	- 2	14.380	17.879	21.378	***
5	- 0	30.248	33.891	37.533	***

5	- 1	30.799	36.243	41.688	***
3	- 9	-53.797	-48.413	-43.028	***
3	- 8	-54.981	-46.995	-39.008	***
3	- 6	-37.933	-34.180	-30.427	***
3	- 7	-36.062	-31.891	-27.719	***
3	- 4	-17.689	-14.076	-10.464	***
3	- 5	-16.443	-12.944	-9.445	***
3	- 2	1.530	4.936	8.341	***
3	- 0	17.394	20.947	24.499	***
3	- 1	17.915	23.300	28.684	***
2	- 9	-58.733	-53.348	-47.963	***
2	- 8	-59.917	-51.930	-43.943	***
2	- 6	-42.869	-39.116	-35.363	***
2	- 7	-40.997	-36.826	-32.655	***
2	- 4	-22.624	-19.012	-15.400	***
2	- 5	-21.378	-17.879	-14.380	***
2	- 3	-8.341	-4.936	-1.530	***
2	- 0	12.459	16.011	19.564	***
2	- 1	12.979	18.364	23.749	***
0	- 9	-74.838	-69.359	-63.880	**

Analysis of Variance Procedure

GROUP Comparison		Lower Confidence Limit	Difference Between Means	Upper Confidence Limit	
0	- 8	-75.992	-67.941	-59.891	***
0	- 6	-59.014	-55.127	-51.240	***
0	- 7	-57.129	-52.837	-48.545	***
0	- 4	-38.774	-35.023	-31.272	***
0	- 5	-37.533	-33.891	-30.248	***
0	- 3	-24.499	-20.947	-17.394	***
0	- 2	-19.564	-16.011	-12.459	***
0	- 1	-3.126	2.353	7.832	
1	- 9	-78.523	-71.712	-64.901	***
1	- 8	-79.304	-70.294	-61.284	***
1	- 6	-63.091	-57.480	-51.869	***
1	- 7	-61.089	-55.190	-49.291	***
1	- 4	-42.894	-37.376	-31.858	***
1	- 5	-41.688	-36.243	-30.799	***
1	- 3	-28.684	-23.300	-17.915	***
1	- 2	-23.749	-18.364	-12.979	***
1	- 0	-7.832	-2.353	3.126	

Analysis of Variance Procedure

T tests (LSD) for variable: PA

NOTE: This test controls the type I comparisonwise error rate not the experimentwise error rate.

Alpha= 0.05 Confidence= 0.95 df= 117 MSE= 8665360
Critical Value of T= 1.98045

Comparisons significant at the 0.05 level are indicated by '***

GROUP Comparison	Lower Confidence Limit	Difference Between Means	Upper Confidence Limit	
0 - 2	-1051.2	872.0	2795.1	
0 - 6	-690.3	1413.7	3517.7	
0 - 4	-114.4	1916.2	3946.8	
0 - 8	-510.1	3848.0	8206.0	
0 - 9	2123.7	5089.6	8055.5	***
0 - 7	2892.7	5216.1	7539.4	***
0 - 3	3425.8	5348.9	7272.1	***
0 - 5	3421.7	5393.4	7365.0	***
0 - 1	2803.3	5769.2	8735.1	***
2 - 0	-2795.1	-872.0	1051.2	
2 - 6	-1489.7	541.8	2573.3	
2 - 4	-911.1	1044.3	2999.6	
2 - 8	-1347.5	2976.0	7299.5	
2 - 9	1302.7	4217.6	7132.5	***
2 - 7	2086.2	4344.1	6602.0	***
2 - 3	2633.4	4477.0	6320.5	***
2 - 5	2627.3	4521.4	6415.5	***
2 - 1	1982.3	4897.2	7812.1	***
6 - 0	-3517.7	-1413.7	690.3	
6 - 2	-2573.3	-541.8	1489.7	
6 - 4	-1631.0	502.5	2636.0	
6 - 8	-1972.7	2434.2	6841.2	
6 - 9	638.6	3675.8	6713.1	***
6 - 7	1388.6	3802.3	6216.1	***
6 - 3	1903.7	3935.2	5966.7	***
6 - 5	1902.2	3979.6	6057.1	***
6 - 1	1318.2	4355.4	7392.7	***
4 - 0	-3946.8	-1916.2	114.4	
4 - 2	-2999.6	-1044.3	911.1	
4 - 6	-2636.0	-502.5	1631.0	
4 - 8	-2440.6	1931.8	6304.1	
4 - 9	186.4	3173.4	6160.3	***
4 - 7	949.8	3299.9	5649.9	***
4 - 3	1477.3	3432.7	5388.1	***
4 - 5	1474.1	3477.1	5480.2	***
4 - 1	866.0	3853.0	6839.9	***

8	- 0	-8206.0	-3848.0	510.1	
8	- 2	-7299.5	-2976.0	1347.5	
8	- 6	-6841.2	-2434.2	1972.7	
8	- 4	-6304.1	-1931.8	2440.6	
8	- 9	-3636.0	1241.6	6119.2	
8	- 7	-3147.7	1368.1	5883.9	
8	- 3	-2822.6	1501.0	5824.5	
8	- 5	-2799.9	1545.4	5890.7	
8	- 1	-2956.4	1921.2	6798.8	
9	- 0	-8055.5	-5089.6	-2123.7	***
9	- 2	-7132.5	-4217.6	-1302.7	***
9	- 6	-6713.1	-3675.8	-638.6	***
9	- 4	-6160.3	-3173.4	-186.4	***
9	- 8	-6119.2	-1241.6	3636.0	
9	- 7	-3066.6	126.5	3319.6	
9	- 3	-2655.6	259.4	3174.3	
9	- 5	-2643.3	303.8	3250.9	
9	- 1	-3007.5	679.6	4366.7	
7	- 0	-7539.4	-5216.1	-2892.7	***
7	- 2	-6602.0	-4344.1	-2086.2	***
7	- 6	-6216.1	-3802.3	-1388.6	***
7	- 4	-5649.9	-3299.9	-940.8	***
7	- 8	-5883.9	-1368.1	3147.7	
7	- 9	-3319.6	-126.5	3066.6	
7	- 3	-2125.0	132.9	2390.7	
7	- 5	-2122.0	177.3	2476.6	
7	- 1	-2640.0	553.1	3746.2	
3	- 0	-7272.1	-5348.9	-3425.8	***
3	- 2	-6320.5	-4477.0	-2633.4	***
3	- 6	-5966.7	-3935.2	-1903.7	***
3	- 4	-5388.1	-3432.7	-1477.3	***
3	- 8	-5824.5	-1501.0	2822.6	
3	- 9	-3174.3	-259.4	2655.6	
3	- 7	-2390.7	-132.9	2125.0	
3	- 5	-1849.6	44.4	1938.5	
3	- 1	-2494.7	420.3	3335.2	
5	- 0	-7365.0	-5393.4	-3421.7	***
5	- 2	-6415.5	-4521.4	-2627.3	***
5	- 6	-6057.1	-3979.6	-1902.2	***
5	- 4	-5480.2	-3477.1	-1474.1	***
5	- 8	-5890.7	-1545.4	2799.9	
5	- 9	-3250.9	-303.8	2643.3	
5	- 7	-2476.6	-177.3	2122.0	
5	- 3	-1938.5	-44.4	1849.6	
5	- 1	-2571.3	375.8	3322.9	
1	- 0	-8735.1	-5769.2	-2803.3	***
1	- 2	-7812.1	-4897.2	-1982.3	***
1	- 6	-7392.7	-4355.4	-1318.2	***
1	- 4	-6839.9	-3853.0	-866.0	***
1	- 8	-6798.8	-1921.2	2956.4	
1	- 9	-4366.7	-679.6	3007.5	
1	- 7	-3746.2	-553.1	2640.0	
1	- 3	-3335.2	-420.3	2494.7	
1	- 5	-3322.9	-375.8	2571.3	

Analysis of Variance Procedure

Dependent Variable: HB

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	120.21442934	13.35715882	2.87	0.0045
Error	105	488.48453892	4.65223370		
Corrected Total	114	608.69896826			

R-Square	C.V.	Root MSE	HB Mean
0.197494	13.99445	2.15690373	15.41256522

Source	DF	Anova SS	Mean Square	F Value	Pr > F
GROUP	9	120.21442934	13.35715882	2.87	0.0045

Analysis of Variance Procedure

T tests (LSD) for variable: HB

NOTE: This test controls the type I comparisonwise error rate not the experimentwise error rate.

Alpha= 0.05 Confidence= 0.95 df= 105 MSE= 4.652234
Critical Value of T= 1.98282

Comparisons significant at the 0.05 level are indicated by '***

GROUP Comparison	Lower Confidence Limit	Difference Between Means	Upper Confidence Limit
7 - 4	-1.3034	0.4785	2.2605
7 - 9	-1.3568	1.0287	3.4141
7 - 6	-0.6032	1.2240	3.0512
7 - 3	-0.5381	1.2249	2.9879

7	- 0	-0.2361	1.6184	3.4729	
7	- 5	0.2228	2.0048	3.7868	***
7	- 2	0.9403	2.6862	4.4322	***
7	- 1	1.7452	4.1307	6.5161	***
7	- 8	1.0146	4.3579	7.7012	***
4	- 7	-2.2605	-0.4785	1.3034	
4	- 9	-1.6411	0.5501	2.7413	
4	- 6	-0.8197	0.7454	2.3106	
4	- 3	-0.7433	0.7464	2.2360	
4	- 0	-0.4571	1.1399	2.7368	
4	- 5	0.0142	1.5262	3.0383	***
4	- 2	0.7383	2.2077	3.6772	***
4	- 1	1.4609	3.6521	5.8433	***
4	- 8	0.6718	3.8794	7.0869	***
9	- 7	-3.4141	-1.0287	1.3568	
9	- 4	-2.7413	-0.5501	1.6411	
9	- 6	-2.0328	0.1953	2.4235	
9	- 3	-1.9795	0.1962	2.3720	
9	- 0	-1.6608	0.5897	2.8403	
9	- 5	-1.2151	0.9761	3.1673	
9	- 2	-0.5044	1.6576	3.8196	
9	- 1	0.3972	3.1020	5.8068	***
9	- 8	-0.2489	3.3293	6.9074	
6	- 7	-3.0512	-1.2240	0.6032	
6	- 4	-2.3106	-0.7454	0.8197	
6	- 9	-2.4235	-0.1953	2.0328	
6	- 3	-1.5426	0.0009	1.5444	
6	- 0	-1.2528	0.3944	2.0417	
6	- 5	-0.7843	0.7808	2.3459	
6	- 2	-0.0617	1.4623	2.9863	
6	- 1	0.6785	2.9067	5.1348	***
6	- 8	-0.0990	3.1339	6.3668	
3	- 7	-2.9879	-1.2249	0.5381	
3	- 4	-2.2360	-0.7464	0.7433	
3	- 9	-2.3720	-0.1962	1.9795	
3	- 6	-1.5444	-0.0009	1.5426	
3	- 0	-1.1822	0.3935	1.9692	
3	- 5	-0.7098	0.7799	2.2695	
3	- 2	0.0150	1.4613	2.9077	***
3	- 1	0.7300	2.9058	5.0815	***
3	- 8	-0.0640	3.1330	6.3301	
0	- 7	-3.4729	-1.6184	0.2361	
0	- 4	-2.7368	-1.1399	0.4571	
0	- 9	-2.8403	-0.5897	1.6608	
0	- 6	-2.0417	-0.3944	1.2528	
0	- 3	-1.9692	-0.3935	1.1822	
0	- 5	-1.2105	0.3864	1.9833	
0	- 2	-0.4888	1.0679	2.6245	
0	- 1	0.2617	2.5123	4.7628	***
0	- 8	-0.5089	2.7395	5.9879	
5	- 7	-3.7868	-2.0048	-0.2228	***

5	- 4	-3.0383	-1.5262	-0.0142	***
5	- 9	-3.1673	-0.9761	1.2151	
5	- 6	-2.3459	-0.7808	0.7843	
5	- 3	-2.2695	-0.7799	0.7098	
5	- 0	-1.9833	-0.3864	1.2105	
5	- 2	-0.7880	0.6815	2.1509	
5	- 1	-0.0653	2.1259	4.3171	
5	- 8	-0.8544	2.3531	5.5607	
2	- 7	-4.4322	-2.6862	-0.9403	***
2	- 4	-3.6772	-2.2077	-0.7383	***
2	- 9	-3.8196	-1.6576	0.5044	
2	- 6	-2.9863	-1.4623	0.0617	
2	- 3	-2.9077	-1.4613	-0.0150	***
2	- 0	-2.6245	-1.0679	0.4888	
2	- 5	-2.1509	-0.6815	0.7880	
2	- 1	-0.7176	1.4444	3.6064	
2	- 8	-1.5160	1.6717	4.8594	
1	- 7	-6.5161	-4.1307	-1.7452	***

Analysis of Variance Procedure

GROUP Comparison		Lower Confidence Limit	Difference Between Means	Upper Confidence Limit	
1	- 4	-5.8433	-3.6521	-1.4609	***
1	- 9	-5.8068	-3.1020	-0.3972	***
1	- 6	-5.1348	-2.9067	-0.6785	***
1	- 3	-5.0815	-2.9058	-0.7300	***
1	- 0	-4.7628	-2.5123	-0.2617	***
1	- 5	-4.3171	-2.1259	0.0653	
1	- 2	-3.6064	-1.4444	0.7176	
1	- 8	-3.3509	0.2273	3.8054	
8	- 7	-7.7012	-4.3579	-1.0146	***
8	- 4	-7.0869	-3.8794	-0.6718	***
8	- 9	-6.9074	-3.3293	0.2489	
8	- 6	-6.3668	-3.1339	0.0990	
8	- 3	-6.3301	-3.1330	0.0640	
8	- 0	-5.9879	-2.7395	0.5089	
8	- 5	-5.5607	-2.3531	0.8544	
8	- 2	-4.8594	-1.6717	1.5160	
8	- 1	-3.8054	-0.2273	3.3509	

Analysis of Variance Procedure

Dependent Variable: L

Source	DF	Sum of Squares	Mean Square

	F Value	Pr > F		
Model		9	119.33223052	13.25913672
	2.08	0.0377		
Error		102	649.18746806	6.36458302
Corrected Total		111	768.51969857	

R-Square	C.V.	Root MSE	L Mean
0.155275	39.52772	2.52281252	6.38238777

Source	DF	Anova SS	Mean Square	F Value	Pr > F
GROUP	9	119.33223052	13.25913672	2.08	0.0377

Analysis of Variance Procedure

T tests (LSD) for variable: L

NOTE: This test controls the type I comparisonwise error rate not the experimentwise error rate.

Alpha= 0.05 Confidence= 0.95 df= 102 MSE= 6.364583
Critical Value of T= 1.98350

Comparisons significant at the 0.05 level are indicated by '***'.

GROUP Comparison	Lower Confidence Limit	Difference Between Means	Upper Confidence Limit	
1 - 0	-2.6024	0.3193	3.2410	
1 - 2	-2.1595	0.6213	3.4021	
1 - 3	-1.3722	1.4086	4.1894	
1 - 5	-0.8190	1.9618	4.7426	
1 - 6	-0.6321	2.2049	5.0419	
1 - 4	-0.4041	2.3932	5.1906	
1 - 7	-0.1844	2.8226	5.8297	
1 - 9	0.4691	3.8259	7.1826	***
1 - 8	0.4793	4.8129	9.1465	***
0 - 1	-3.2410	-0.3193	2.6024	
0 - 2	-1.6343	0.3020	2.2383	

0	- 3	-0.8470	1.0893	3.0256	
0	- 5	-0.2938	1.6425	3.5788	
0	- 6	-0.1305	1.8856	3.9018	
0	- 4	0.1140	2.0740	4.0339	***
0	- 7	0.2542	2.5034	4.7525	***
0	- 9	0.8076	3.5066	6.2055	***
0	- 8	0.6470	4.4936	8.3402	***
2	- 1	-3.4021	-0.6213	2.1595	
2	- 0	-2.2383	-0.3020	1.6343	
2	- 3	-0.9291	0.7873	2.5037	
2	- 5	-0.3759	1.3404	3.0568	
2	- 6	-0.2224	1.5836	3.3895	
2	- 4	0.0290	1.7719	3.5149	***
2	- 7	0.1385	2.2013	4.2641	***
2	- 9	0.6588	3.2045	5.7503	***
2	- 8	0.4509	4.1916	7.9323	***
3	- 1	-4.1894	-1.4086	1.3722	
3	- 0	-3.0256	-1.0893	0.8470	
3	- 2	-2.5037	-0.7873	0.9291	
3	- 5	-1.1632	0.5531	2.2695	
3	- 6	-1.0097	0.7963	2.6022	
3	- 4	-0.7583	0.9846	2.7276	
3	- 7	-0.6488	1.4140	3.4768	
3	- 9	-0.1285	2.4172	4.9630	
3	- 8	-0.3364	3.4043	7.1450	
5	- 1	-4.7426	-1.9618	0.8190	
5	- 0	-3.5788	-1.6425	0.2938	
5	- 2	-3.0568	-1.3404	0.3759	
5	- 3	-2.2695	-0.5531	1.1632	
5	- 6	-1.5628	0.2431	2.0491	
5	- 4	-1.3115	0.4315	2.1745	
5	- 7	-1.2019	0.8609	2.9237	
5	- 9	-0.6817	1.8641	4.4099	
5	- 8	-0.8896	2.8511	6.5918	
6	- 1	-5.0419	-2.2049	0.6321	
6	- 0	-3.9018	-1.8856	0.1305	
6	- 2	-3.3895	-1.5836	0.2224	
6	- 3	-2.6022	-0.7963	1.0097	
6	- 5	-2.0491	-0.2431	1.5628	
6	- 4	-1.6429	0.1883	2.0196	
6	- 7	-1.5202	0.6177	2.7557	
6	- 9	-0.9861	1.6210	4.2280	
6	- 8	-1.1747	2.6080	6.3907	
4	- 1	-5.1906	-2.3932	0.4041	
4	- 0	-4.0339	-2.0740	-0.1140	***
4	- 2	-3.5149	-1.7719	-0.0290	***
4	- 3	-2.7276	-0.9846	0.7583	
4	- 5	-2.1745	-0.4315	1.3115	
4	- 6	-2.0196	-0.1883	1.6429	
4	- 7	-1.6556	0.4294	2.5144	
4	- 9	-1.1312	1.4326	3.9964	
4	- 8	-1.3333	2.4197	6.1726	

7	- 1	-5.8297	-2.8226	0.1844	
7	- 0	-4.7525	-2.5034	-0.2542	***
7	- 2	-4.2641	-2.2013	-0.1385	***
7	- 3	-3.4768	-1.4140	0.6488	
7	- 5	-2.9237	-0.8609	1.2019	
7	- 6	-2.7557	-0.6177	1.5202	
7	- 4	-2.5144	-0.4294	1.6556	
7	- 9	-1.7879	1.0032	3.7943	
7	- 8	-1.9215	1.9903	5.9020	
9	- 1	-7.1826	-3.8259	-0.4691	***

Analysis of Variance Procedure

GROUP Comparison		Lower Confidence Limit	Difference Between Means	Upper Confidence Limit	
9	- 0	-6.2055	-3.5066	-0.8076	***
9	- 2	-5.7503	-3.2045	-0.6588	***
9	- 3	-4.9630	-2.4172	0.1285	
9	- 5	-4.4099	-1.8641	0.6817	
9	- 6	-4.2280	-1.6210	0.9861	
9	- 4	-3.9964	-1.4326	1.1312	
9	- 7	-3.7943	-1.0032	1.7879	
9	- 8	-3.1996	0.9870	5.1737	
8	- 1	-9.1465	-4.8129	-0.4793	***
8	- 0	-8.3402	-4.4936	-0.6470	***
8	- 2	-7.9323	-4.1916	-0.4509	***
8	- 3	-7.1450	-3.4043	0.3364	
8	- 5	-6.5918	-2.8511	0.8896	
8	- 6	-6.3907	-2.6080	1.1747	
8	- 4	-6.1726	-2.4197	1.3333	
8	- 7	-5.9020	-1.9903	1.9215	
8	- 9	-5.1737	-0.9870	3.1996	

Analysis of Variance Procedure

Dependent Variable: B

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	88.12238350	9.79137594	2.00	0.0482
Error	91	445.80073965	4.89890923		
Corrected Total	100	533.92312315			

R-Square	C.V.	Root MSE	B Mean
0.165047	40.32171	2.21334797	5.48922203

Source	DF	Anova SS	Mean Square	F Value	Pr > F
GROUP	9	88.12238350	9.79137594	2.00	0.0482

Analysis of Variance Procedure

T tests (LSD) for variable: B

NOTE: This test controls the type I comparisonwise error rat
e not the
experimentwise error rate.

Alpha= 0.05 Confidence= 0.95 df= 91 MSE= 4.898909
Critical Value of T= 1.98638

Comparisons significant at the 0.05 level are indicated by '***

GROUP Comparison	Lower Confidence Limit	Difference Between Means	Upper Confidence Limit	
0 - 2	-1.5656	0.2058	1.9772	
0 - 1	-1.9305	0.4408	2.8122	
0 - 3	-0.4763	1.2457	2.9677	
0 - 5	-0.3655	1.4356	3.2368	
0 - 6	-0.1370	1.6641	3.4653	
0 - 7	-0.1551	1.8878	3.9307	
0 - 4	0.1680	1.9132	3.6585	***
0 - 9	0.9967	3.5638	6.1308	***
0 - 8	0.9347	4.3144	7.6940	***
2 - 0	-1.9772	-0.2058	1.5656	
2 - 1	-2.0555	0.2350	2.5256	
2 - 3	-0.5691	1.0399	2.6488	
2 - 5	-0.4636	1.2298	2.9232	
2 - 6	-0.2351	1.4583	3.1517	
2 - 7	-0.2666	1.6819	3.6305	
2 - 4	0.0736	1.7074	3.3412	***
2 - 9	0.8653	3.3579	5.8505	***
2 - 8	0.7851	4.1086	7.4320	***
1 - 0	-2.8122	-0.4408	1.9305	
1 - 2	-2.5256	-0.2350	2.0555	
1 - 3	-1.4477	0.8049	3.0574	
1 - 5	-1.3188	0.9948	3.3084	
1 - 6	-1.0903	1.2233	3.5369	
1 - 7	-1.0595	1.4469	3.9533	
1 - 4	-0.7980	1.4724	3.7427	
1 - 9	0.1736	3.1229	6.0722	***
1 - 8	0.1951	3.8735	7.5520	***
3 - 0	-2.9677	-1.2457	0.4763	
3 - 2	-2.6488	-1.0399	0.5691	
3 - 1	-3.0574	-0.8049	1.4477	

3	- 5	-1.4517	0.1899	1.8316	
3	- 6	-1.2232	0.4184	2.0601	
3	- 7	-1.2617	0.6421	2.5458	
3	- 4	-0.9126	0.6675	2.2476	
3	- 9	-0.1397	2.3181	4.7758	
3	- 8	-0.2287	3.0687	6.3661	
5	- 0	-3.2368	-1.4356	0.3655	
5	- 2	-2.9232	-1.2298	0.4636	
5	- 1	-3.3084	-0.9948	1.3188	
5	- 3	-1.8316	-0.1899	1.4517	
5	- 6	-1.4960	0.2285	1.9530	
5	- 7	-1.5235	0.4521	2.4278	
5	- 4	-1.1884	0.4776	2.1436	
5	- 9	-0.3857	2.1281	4.6419	
5	- 8	-0.4607	2.8788	6.2182	
6	- 0	-3.4653	-1.6641	0.1370	
6	- 2	-3.1517	-1.4583	0.2351	
6	- 1	-3.5369	-1.2233	1.0903	
6	- 3	-2.0601	-0.4184	1.2232	
6	- 5	-1.9530	-0.2285	1.4960	
6	- 7	-1.7520	0.2236	2.1993	
6	- 4	-1.4169	0.2491	1.9151	
6	- 9	-0.6142	1.8996	4.4135	
6	- 8	-0.6892	2.6503	5.9897	
7	- 0	-3.9307	-1.8878	0.1551	
7	- 2	-3.6305	-1.6819	0.2666	
7	- 1	-3.9533	-1.4469	1.0595	
7	- 3	-2.5458	-0.6421	1.2617	
7	- 5	-2.4278	-0.4521	1.5235	
7	- 6	-2.1993	-0.2236	1.7520	
7	- 4	-1.8993	0.0255	1.9503	
7	- 9	-1.0163	1.6760	4.3683	
7	- 8	-1.0491	2.4266	5.9024	
4	- 0	-3.6585	-1.9132	-0.1680	***
4	- 2	-3.3412	-1.7074	-0.0736	***
4	- 1	-3.7427	-1.4724	0.7980	
4	- 3	-2.2476	-0.6675	0.9126	
4	- 5	-2.1436	-0.4776	1.1884	
4	- 6	-1.9151	-0.2491	1.4169	
4	- 7	-1.9503	-0.0255	1.8993	
4	- 9	-0.8235	1.6505	4.1246	
4	- 8	-0.9084	2.4012	5.7108	
9	- 0	-6.1308	-3.5638	-0.9967	***

Analysis of Variance Procedure

GROUP Comparison	Lower Confidence Limit	Difference Between Means	Upper Confidence Limit

9	- 2	-5.8505	-3.3579	-0.8653	***
9	- 1	-6.0722	-3.1229	-0.1736	***
9	- 3	-4.7758	-2.3181	0.1397	
9	- 5	-4.6419	-2.1281	0.3857	
9	- 6	-4.4135	-1.8996	0.6142	
9	- 7	-4.3683	-1.6760	1.0163	
9	- 4	-4.1246	-1.6505	0.8235	
9	- 8	-3.0569	0.7506	4.5581	
8	- 0	-7.6940	-4.3144	-0.9347	***
8	- 2	-7.4320	-4.1086	-0.7851	***
8	- 1	-7.5520	-3.8735	-0.1951	***
8	- 3	-6.3661	-3.0687	0.2287	
8	- 5	-6.2182	-2.8788	0.4607	
8	- 6	-5.9897	-2.6503	0.6892	
8	- 7	-5.9024	-2.4266	1.0491	
8	- 4	-5.7108	-2.4012	0.9084	
8	- 9	-4.5581	-0.7506	3.0569	

Analysis of Variance Procedure

Dependent Variable: TEMP

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	72220134835.20500000	8024459426.13389000	0.78	0.6389
Error	70	723903158047.34500000	10341473686.39060000		
Corrected Total	79	796123292882.55000000			

R-Square	C.V.	Root MSE	TEMP Mean
0.090715	-526.2708	101693.03656785	-19323.32858403

Source	DF	Anova SS	Mean Square	F Value	Pr > F
GROUP	9	72220134835.20510000	8024459426.13390000	0.78	0.6389

Analysis of Variance Procedure

Dependent Variable: LNTEMP

Source	DF	Sum of Squares	Mean Square

	F Value	Pr > F		
Model		9	53259480.79518490	5917720.08835389
	1.01	0.4443		
Error		70	412107137.98974400	5887244.82842492
Corrected Total		79	465366618.78492900	

R-Square	C.V.	Root MSE	LNTEMP Mean
0.114446	-549.7364	2426.36452917	-441.36869358

Source	DF	Anova SS	Mean Square	F Value	Pr > F
GROUP	9	53259480.79518490	5917720.08835389	1.01	0.4443

Analysis of Variance Procedure

Dependent Variable: LNTEMP

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	53259480.79518490	5917720.08835389	1.01	0.4443
Error	70	412107137.98974400	5887244.82842492		
Corrected Total	79	465366618.78492900			

R-Square	C.V.	Root MSE	LNTEMP Mean
0.114446	-549.7364	2426.36452917	-441.36869358

Source	DF	Anova SS	Mean Square	F Value	Pr > F
GROUP	9	53259480.79518490	5917720.08835389	1.01	0.4443

Analysis of Variance Procedure

T tests (LSD) for variable: TEMP

NOTE: This test controls the type I comparisonwise error rat
e not the
experimentwise error rate.

Alpha= 0.05 Confidence= 0.95 df= 70 MSE= 1.034E10
Critical Value of T= 1.99444

Comparisons significant at the 0.05 level are indicated by '***

GROUP Comparison	Lower Confidence Limit	Difference Between Means	Upper Confidence Limit
5 - 7	-104342	2554	109450
5 - 2	-77387	10562	98511
5 - 0	-49395	40041	129476
5 - 6	-47847	40102	128051
5 - 1	-76928	44952	166832
5 - 8	-166167	47624	261416
5 - 3	-37963	60590	159143
5 - 9	-32442	89438	211317
5 - 4	-3299	89890	183080
7 - 5	-109450	-2554	104342
7 - 2	-92094	8008	108109
7 - 0	-63923	37487	138897
7 - 6	-62554	37548	137650
7 - 1	-88522	42398	173318
7 - 8	-174001	45070	264141
7 - 3	-51500	58036	167571
7 - 9	-44036	86884	217803
7 - 4	-17400	87336	192072

2	- 5	-98511	-10562	77387
2	- 7	-108109	-8008	92094
2	- 0	-51714	29479	110672
2	- 6	-50012	29540	109093
2	- 1	-81577	34390	150357
2	- 8	-173414	37062	247539
2	- 3	-41111	50028	141167
2	- 9	-37091	78876	194843
2	- 4	-5982	79329	164639
0	- 5	-129476	-40041	49395
0	- 7	-138897	-37487	63923
0	- 2	-110672	-29479	51714
0	- 6	-81132	61	81255
0	- 1	-112187	4911	122009
0	- 8	-203519	7583	218686
0	- 3	-72025	20549	113123
0	- 9	-67702	49397	166495
0	- 4	-36993	49850	136692
6	- 5	-128051	-40102	47847
6	- 7	-137650	-37548	62554
6	- 2	-109093	-29540	50012
6	- 0	-81255	-61	81132
6	- 1	-111117	4850	120816
6	- 8	-202955	7522	217999
6	- 3	-70651	20488	111627
6	- 9	-66632	49335	165302
6	- 4	-35523	49788	135099
1	- 5	-166832	-44952	76928
1	- 7	-173318	-42398	88522
1	- 2	-150357	-34390	81577
1	- 0	-122009	-4911	112187
1	- 6	-120816	-4850	111117
1	- 8	-224088	2672	229433
1	- 3	-108563	15638	139840
1	- 9	-98930	44486	187902
1	- 4	-75051	44939	164929
8	- 5	**-261416**	**-47624**	**166167**

8	- 7	-264141	-45070	174001
8	- 2	-247539	-37062	173414
8	- 0	-218686	-7583	203519
8	- 6	-217999	-7522	202955
8	- 1	-229433	-2672	224088
8	- 3	-202158	12966	228089
8	- 9	-184947	41813	268573
8	- 4	-170454	42266	254986
3	- 5	-159143	-60590	37963
3	- 7	-167571	-58036	51500
3	- 2	-141167	-50028	41111
3	- 0	-113123	-20549	72025
3	- 6	-111627	-20488	70651
3	- 1	-139840	-15638	108563
3	- 8	-228089	-12966	202158
3	- 9	-95354	28848	153049
3	- 4	-66906	29301	125507
9	- 5	-211317	-89438	32442

Analysis of Variance Procedure

GROUP Comparison		Lower Confidence Limit	Difference Between Means	Upper Confidence Limit
9	- 7	-217803	-86884	44036
9	- 2	-194843	-78876	37091
9	- 0	-166495	-49397	67702
9	- 6	-165302	-49335	66632
9	- 1	-187902	-44486	98930
9	- 8	-268573	-41813	184947
9	- 3	-153049	-28848	95354
9	- 4	-119537	453	120443
4	- 5	-183080	-89890	3299
4	- 7	-192072	-87336	17400
4	- 2	-164639	-79329	5982
4	- 0	-136692	-49850	36993
4	- 6	-135099	-49788	35523
4	- 1	-164929	-44939	75051
4	- 8	-254986	-42266	170454
4	- 3	-125507	-29301	66906
4	- 9	-120443	-453	119537

Analysis of Variance Procedure

T tests (LSD) for variable: LNTEMP

NOTE: This test controls the type I comparisonwise error rat

e not the

experimentwise error rate.

Alpha= 0.05 Confidence= 0.95 df= 70 MSE= 5887245
Critical Value of T= 1.99444

Comparisons significant at the 0.05 level are indicated by '***'.

GROUP Comparison		Lower Confidence Limit	Difference Between Means	Upper Confidence Limit	
7	- 5	-2356.7	193.8	2744.3	
7	- 2	-2106.5	281.9	2670.3	
7	- 0	-1563.5	856.1	3275.7	
7	- 6	-1428.4	960.0	3348.4	
7	- 1	-2095.8	1027.9	4151.6	
7	- 8	-3980.6	1246.4	6473.3	
7	- 3	-945.0	1668.5	4282.0	
7	- 4	-132.0	2367.0	4866.0	
7	- 9	-381.6	2742.1	5865.8	
5	- 7	-2744.3	-193.8	2356.7	
5	- 2	-2010.3	88.1	2186.5	
5	- 0	-1471.6	662.3	2796.2	
5	- 6	-1332.2	766.2	2864.6	
5	- 1	-2073.9	834.1	3742.1	
5	- 8	-4048.4	1052.6	6153.6	
5	- 3	-876.8	1474.7	3826.1	
5	- 4	-50.3	2173.2	4396.7	
5	- 9	-359.7	2548.3	5456.4	
2	- 7	-2670.3	-281.9	2106.5	
2	- 5	-2186.5	-88.1	2010.3	
2	- 0	-1363.0	574.2	2511.5	
2	- 6	-1220.0	678.1	2576.2	
2	- 1	-2021.0	746.0	3512.9	
2	- 8	-4057.5	964.5	5986.4	
2	- 3	-788.0	1386.6	3561.1	
2	- 4	49.6	2085.1	4120.6	***
2	- 9	-306.7	2460.2	5227.2	
0	- 7	-3275.7	-856.1	1563.5	
0	- 5	-2796.2	-662.3	1471.6	
0	- 2	-2511.5	-574.2	1363.0	
0	- 6	-1833.3	103.9	2041.1	
0	- 1	-2622.2	171.8	2965.7	
0	- 8	-4646.6	390.2	5427.1	
0	- 3	-1396.4	812.4	3021.2	
0	- 4	-561.2	1510.9	3582.9	
0	- 9	-907.9	1886.0	4679.9	
6	- 7	-3348.4	-960.0	1428.4	
6	- 5	-2864.6	-766.2	1332.2	
6	- 2	-2576.2	-678.1	1220.0	
6	- 0	-2041.1	-103.9	1833.3	

6	- 1	-2699.1	67.9	2834.8
6	- 8	-4735.6	286.3	5308.3
6	- 3	-1466.1	708.5	2883.0
6	- 4	-628.5	1407.0	3442.5
6	- 9	-984.8	1782.1	4549.1
1	- 7	-4151.6	-1027.9	2095.8
1	- 5	-3742.1	-834.1	2073.9
1	- 2	-3512.9	-746.0	2021.0
1	- 0	-2965.7	-171.8	2622.2
1	- 6	-2834.8	-67.9	2699.1
1	- 8	-5191.9	218.5	5628.9
1	- 3	-2322.8	640.6	3604.0
1	- 4	-1523.8	1339.1	4202.0
1	- 9	-1707.6	1714.3	5136.1
8	- 7	-6473.3	-1246.4	3980.6
8	- 5	-6153.6	-1052.6	4048.4
8	- 2	-5986.4	-964.5	4057.5
8	- 0	-5427.1	-390.2	4646.6
8	- 6	-5308.3	-286.3	4735.6
8	- 1	-5628.9	-218.5	5191.9
8	- 3	-4710.7	422.1	5554.9
8	- 4	-3954.8	1120.6	6196.1
8	- 9	-3914.7	1495.8	6906.2
3	- 7	-4282.0	-1668.5	945.0
3	- 5	-3826.1	-1474.7	876.8
3	- 2	-3561.1	-1386.6	788.0
3	- 0	-3021.2	-812.4	1396.4
3	- 6	-2883.0	-708.5	1466.1
3	- 1	-3604.0	-640.6	2322.8
3	- 8	-5554.9	-422.1	4710.7
3	- 4	-1596.9	698.5	2994.0
3	- 9	-1889.8	1073.7	4037.1
4	- 7	-4866.0	-2367.0	132.0

Analysis of Variance Procedure

GROUP Comparison		Lower Confidence Limit	Difference Between Means	Upper Confidence Limit	
4	- 5	-4396.7	-2173.2	50.3	
4	- 2	-4120.6	-2085.1	-49.6	***
4	- 0	-3582.9	-1510.9	561.2	
4	- 6	-3442.5	-1407.0	628.5	
4	- 1	-4202.0	-1339.1	1523.8	
4	- 8	-6196.1	-1120.6	3954.8	
4	- 3	-2994.0	-698.5	1596.9	
4	- 9	-2487.8	375.1	3238.1	
9	- 7	-5865.8	-2742.1	381.6	
9	- 5	-5456.4	-2548.3	359.7	
9	- 2	-5227.2	-2460.2	306.7	
9	- 0	-4679.9	-1886.0	907.9	
9	- 6	-4549.1	-1782.1	984.8	
9	- 1	-5136.1	-1714.3	1707.6	
9	- 8	-6906.2	-1495.8	3914.7	
9	- 3	-4037.1	-1073.7	1889.8	
9	- 4	-3238.1	-375.1	2487.8	

Analysis of Variance Procedure
Class Level Information

Class	Levels	Values
GROUP	10	0 1 2 3 4 5 6 7 8 9
AI	2	0 1
MF	2	0 1
AGE20	5	0 1 2 3 4

Number of observations in data set = 130

Group	Obs	Dependent Variables
1	96	INVSL
2	127	AGE PA
3	115	HB
4	112	L
5	101	B
6	80	TEMP LNTEMP

NOTE: Variables in each group are consistent with respect to the presence or absence of missing values.

Analysis of Variance Procedure

Dependent Variable: INVSL

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.73949651	0.73949651	13.77	0.0003
Error	94	5.04731992	0.05369489		
Corrected Total	95	5.78681643			

R-Square	C.V.	Root MSE	INVSL Mean
0.127790	41.45569	0.23172158	0.55896207

Source	DF	Anova SS	Mean Square	F Value	Pr > F
AI	1	0.73949651	0.73949651	13.77	0.0003

Analysis of Variance Procedure

T tests (LSD) for variable: INVSL

NOTE: This test controls the type I comparisonwise error rate not the experimentwise error rate.

Alpha= 0.05 df= 94 MSE= 0.053695
Critical Value of T= 1.99
Least Significant Difference= 0.0941
WARNING: Cell sizes are not equal.
Harmonic Mean of cell sizes= 47.8125

Means with the same letter are not significantly different.

T Grouping	Mean	N	AI
A	0.64141	51	0
B	0.46553	45	1

Analysis of Variance Procedure

Dependent Variable: AGE

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	1418.95324338	1418.95324338	3.32	0.0706
Error	125	53351.06398011	426.80851184		
Corrected Total	126	54770.01722350			

R-Square	C.V.	Root MSE	AGE Mean
0.025907	47.44905	20.65934442	43.54006299

Source	DF	Anova SS	Mean Square	F Value	Pr > F
AI	1	1418.95324338	1418.95324338	3.32	0.0706

Analysis of Variance Procedure

Dependent Variable: PA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	571384763.14025900	571384763.14025900	67.13	0.0001
Error	125	1064026096.05202000	8512208.76841619		
Corrected Total	126	1635410859.19228000			

R-Square	C.V.	Root MSE	PA Mean
0.349383	102.0910	2917.56898263	2857.81181102

Source	DF	Anova SS	Mean Square	F Value	Pr > F

AI	1	571384763.14025900	571384763.14025900	67.13 0.0001

Analysis of Variance Procedure

T tests (LSD) for variable: AGE

NOTE: This test controls the type I comparisonwise error rate not the experimentwise error rate.

Alpha= 0.05 df= 125 MSE= 426.8085
Critical Value of T= 1.98
Least Significant Difference= 7.2837
WARNING: Cell sizes are not equal.
Harmonic Mean of cell sizes= 63.02362

Means with the same letter are not significantly different.

T Grouping	Mean	N	AI
A	47.186	58	1
A			
A	40.475	69	0

Analysis of Variance Procedure

T tests (LSD) for variable: PA

NOTE: This test controls the type I comparisonwise error rat
e not the
experimentwise error rate.

Alpha= 0.05 df= 125 MSE= 8512209
Critical Value of T= 1.98
Least Significant Difference= 1028.6
WARNING: Cell sizes are not equal.
Harmonic Mean of cell sizes= 63.02362

Means with the same letter are not significantly different.

T Grouping	Mean	N	AI
A	4802.5	69	0
B	544.3	58	1

Analysis of Variance Procedure

Dependent Variable: HB

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.40977403	0.40977403	0.08	0.7831
Error	113	608.28919423	5.38309021		
Corrected Total	114	608.69896826			

R-Square	C.V.	Root MSE	HB Mean
0.000673	15.05362	2.32014875	15.41256522

Source	DF	Anova SS	Mean Square	F Value	Pr > F
AI	1	0.40977403	0.40977403	0.08	0.7831

Analysis of Variance Procedure

T tests (LSD) for variable: HB

NOTE: This test controls the type I comparisonwise error rat
e not the
experimentwise error rate.

Alpha= 0.05 df= 113 MSE= 5.38309
Critical Value of T= 1.98
Least Significant Difference= 0.8612
WARNING: Cell sizes are not equal.
Harmonic Mean of cell sizes= 56.97391

Means with the same letter are not significantly different.

T Grouping	Mean	N	AI
A	15.4783	52	1
A			
A	15.3583	63	0

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Analysis of Variance Procedure

Dependent Variable: L

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	4.69598500	4.69598500	0.68	0.4126
Error	110	763.82371358	6.94385194		
Corrected Total	111	768.51969857			

R-Square	C.V.	Root MSE	L Mean
0.006110	41.28735	2.63511896	6.38238777

Source	DF	Anova SS	Mean Square	F Value	Pr > F

AI	1	4.69598500	4.69598500
0.68	0.4126		

Analysis of Variance Procedure

T tests (LSD) for variable: L

NOTE: This test controls the type I comparisonwise error rate not the experimentwise error rate.

Alpha= 0.05 df= 110 MSE= 6.943852
Critical Value of T= 1.98
Least Significant Difference= 0.9894
WARNING: Cell sizes are not equal.
Harmonic Mean of cell sizes= 55.71429

Means with the same letter are not significantly different.

T Grouping	Mean	N	AI
A	6.5730	60	0
A			
A	6.1624	52	1

Analysis of Variance Procedure

Dependent Variable: B

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	4.02230278	4.02230278	0.75	0.3881
Error	99	529.90082037	5.35253354		
Corrected Total	100	533.92312315			

R-Square	C.V.	Root MSE	B Mean
0.007533	42.14722	2.31355431	5.48922203

Source	DF	Anova SS	Mean Square

	F Value	Pr > F		
AI	1	4.02230278	4.02230278	
	0.75	0.3881		

Analysis of Variance Procedure

T tests (LSD) for variable: B

NOTE: This test controls the type I comparisonwise error rate not the experimentwise error rate.

Alpha= 0.05 df= 99 MSE= 5.352534
Critical Value of T= 1.98
Least Significant Difference= 0.9172
WARNING: Cell sizes are not equal.
Harmonic Mean of cell sizes= 50.09901

Means with the same letter are not significantly different.

T Grouping	Mean	N	AI
A	5.6717	55	0
A			
A	5.2710	46	1

Analysis of Variance Procedure

Dependent Variable: B

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	4.02230278	4.02230278	0.75	0.3881
Error	99	529.90082037	5.35253354		
Corrected Total	100	533.92312315			

R-Square	C.V.	Root MSE	B Mean
0.007533	42.14722	2.31355431	5.48922203

Source	DF	Anova SS	Mean Square	F Value	Pr > F
AI	1	4.02230278	4.02230278	0.75	0.3881

Analysis of Variance Procedure

T tests (LSD) for variable: B

NOTE: This test controls the type I comparisonwise error rate not the experimentwise error rate.

Alpha= 0.05 df= 99 MSE= 5.352534
Critical Value of T= 1.98
Least Significant Difference= 0.9172
WARNING: Cell sizes are not equal.
Harmonic Mean of cell sizes= 50.09901

Means with the same letter are not significantly different.

T Grouping	Mean	N	AI
A	5.6717	55	0
A			
A	5.2710	46	1

Analysis of Variance Procedure

Dependent Variable: TEMP

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	1570047533.52539000	1570047533.52539000	0.15	0.6957
Error	78	794553245349.02500000	10186580068.57720000		
Corrected Total	79	796123292882.55000000			

R-Square	C.V.	Root MSE	TEMP Mean

Means with the same letter are not significantly different.

T Grouping	Mean	N	AI
A	5.6717	55	0
A			
A	5.2710	46	1

Analysis of Variance Procedure

Dependent Variable: TEMP

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	1570047533.52539000	1570047533.52539000	0.15	0.6957
Error	78	794553245349.02500000	10186580068.57720000		
Corrected Total	79	796123292882.55000000			

R-Square	C.V.	Root MSE	TEMP Mean
0.001972	-522.3147	100928.58895564	-19323.32858403

Source	DF	Anova SS	Mean Square	F Value	Pr > F

AI		1	1570047533.52539000	1570047533.52539000
0.15	0.6957			

Analysis of Variance Procedure

Dependent Variable: LNTEMP

Source	DF	Sum of Squares	Mean Square
F Value	Pr > F		
Model	1	104980.33199513	104980.33199513
0.02	0.8948		
Error	78	465261638.45293400	5964892.80067865
Corrected Total	79	465366618.78492900	

R-Square	C.V.	Root MSE
LNTEMP Mean		
0.000226	-553.3498	2442.31300219
-441.36869358		

Source	DF	Anova SS	Mean Square
F Value	Pr > F		
AI	1	104980.33199511	104980.33199511
0.02	0.8948		

Analysis of Variance Procedure

T tests (LSD) for variable: TEMP

NOTE: This test controls the type I comparisonwise error rat
e not the
experimentwise error rate.

Alpha= 0.05 df= 78 MSE= 1.019E10
Critical Value of T= 1.99
Least Significant Difference= 46112
WARNING: Cell sizes are not equal.
Harmonic Mean of cell sizes= 37.975

Means with the same letter are not significantly different.

T Grouping	Mean	N	AI
A	-13754	31	1
A			
A	-22847	49	0

Analysis of Variance Procedure

T tests (LSD) for variable: LNTEMP

NOTE: This test controls the type I comparisonwise error rat
e not the
experimentwise error rate.

Alpha= 0.05 df= 78 MSE= 5964893
Critical Value of T= 1.99
Least Significant Difference= 1115.8
WARNING: Cell sizes are not equal.
Harmonic Mean of cell sizes= 37.975

Means with the same letter are not significantly different.

T Grouping	Mean	N	AI
A	-395.8	31	1
A			
A	-470.2	49	0

Analysis of Variance Procedure
Class Level Information

Class	Levels	Values
GROUP	10	0 1 2 3 4 5 6 7 8 9
AI	2	0 1
MF	2	0 1
AGE20	5	0 1 2 3 4

Number of observations in data set = 130

Group	Obs	Dependent Variables
1	96	INVSL
2	127	AGE PA
3	115	HB
4	112	L
5	101	B

6 80 TEMP LNTEMP

NOTE: Variables in each group are consistent with respect to the presence or absence of missing values.

Analysis of Variance Procedure

Dependent Variable: INVSL

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.01722547	0.01722547	0.28	0.5975
Error	94	5.76959095	0.06137863		
Corrected Total	95	5.78681643			

R-Square	C.V.	Root MSE	INVSL Mean
0.002977	44.32270	0.24774710	0.55896207

Source	DF	Anova SS	Mean Square	F Value	Pr > F
MF	1	0.01722547	0.01722547	0.28	0.5975

Analysis of Variance Procedure

T tests (LSD) for variable: INVSL

NOTE: This test controls the type I comparisonwise error rate not the experimentwise error rate.

Alpha= 0.05 df= 94 MSE= 0.061379
Critical Value of T= 1.99
Least Significant Difference= 0.1027
WARNING: Cell sizes are not equal.
Harmonic Mean of cell sizes= 45.91667

Means with the same letter are not significantly different.

T Grouping	Mean	N	MF
A	0.56980	58	0
A			
A	0.54241	38	1

Analysis of Variance Procedure

Dependent Variable: AGE

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	593.82272274	593.82272274	1.37	0.2440
Error	125	54176.19450076	433.40955601		
Corrected Total	126	54770.01722350			

R-Square	C.V.	Root MSE	AGE Mean
0.010842	47.81456	20.81849072	43.54006299

Source	DF	Anova SS	Mean Square	F Value	Pr > F
MF	1	593.82272274	593.82272274	1.37	0.2440

Analysis of Variance Procedure

Dependent Variable: PA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	232749.81389761	232749.81389761	0.02	0.8941
Error	125	1635178109.37838000	13081424.87502700		
Corrected Total	126	1635410859.19228000			

R-Square	C.V.	Root MSE	PA Mean
0.000142	126.5593	3616.82524806	2857.81181102

Source	DF	Anova SS	Mean Square	F Value	Pr > F
MF	1	232749.81389773	232749.81389773	0.02	0.8941

Analysis of Variance Procedure

T tests (LSD) for variable: AGE

NOTE: This test controls the type I comparisonwise error rate not the experimentwise error rate.

Alpha= 0.05 df= 125 MSE= 433.4096
Critical Value of T= 1.98
Least Significant Difference= 7.5723
WARNING: Cell sizes are not equal.
Harmonic Mean of cell sizes= 59.2126

Means with the same letter are not significantly different.

T Grouping	Mean	N	MF
A	45.197	80	0
A			
A	40.719	47	1

Analysis of Variance Procedure

T tests (LSD) for variable: PA

NOTE: This test controls the type I comparisonwise error rate not the experimentwise error rate.

Alpha= 0.05 df= 125 MSE= 13081425
Critical Value of T= 1.98
Least Significant Difference= 1315.6
WARNING: Cell sizes are not equal.

Harmonic Mean of cell sizes= 59.2126

Means with the same letter are not significantly different.

T Grouping	Mean	N	MF
A	2913.7	47	1
A			
A	2825.0	80	0

Analysis of Variance Procedure

Dependent Variable: HB

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	103.38705522	103.38705522	23.12	0.0001
Error	113	505.31191304	4.47178684		
Corrected Total	114	608.69896826			

R-Square	C.V.	Root MSE	HB Mean
0.169849	13.72036	2.11465998	15.41256522

Source	DF	Anova SS	Mean Square	F Value	Pr > F
MF	1	103.38705522	103.38705522	23.12	0.0001

Analysis of Variance Procedure

T tests (LSD) for variable: HB

NOTE: This test controls the type I comparisonwise error rate not the experimentwise error rate.

Alpha= 0.05 df= 113 MSE= 4.471787
Critical Value of T= 1.98
Least Significant Difference= 0.7975
WARNING: Cell sizes are not equal.
Harmonic Mean of cell sizes= 55.2

Means with the same letter are not significantly different.

T Grouping	Mean	N	MF
A	16.1867	69	0
B	14.2513	46	1

Analysis of Variance Procedure

Dependent Variable: L

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	23.97583445	23.97583445	3.54	0.0625
Error	110	744.54386413	6.76858058		
Corrected Total	111	768.51969857			

R-Square	C.V.	Root MSE	L Mean
0.031197	40.76295	2.60164959	6.38238777

Source	DF	Anova SS	Mean Square	F Value	Pr > F
MF	1	23.97583445	23.97583445	3.54	0.0625

Analysis of Variance Procedure

T tests (LSD) for variable: L

NOTE: This test controls the type I comparisonwise error rat
e not the
experimentwise error rate.

Alpha= 0.05 df= 110 MSE= 6.768581
Critical Value of T= 1.98
Least Significant Difference= 1.0017
WARNING: Cell sizes are not equal.
Harmonic Mean of cell sizes= 52.98214

Means with the same letter are not significantly different.

T Grouping	Mean	N	MF
A	6.7476	69	0
A			
A	5.7963	43	1

Analysis of Variance Procedure

Dependent Variable: B

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	24.09276868	24.09276868	4.68	0.0330
Error	99	509.83035447	5.14980156		
Corrected Total	100	533.92312315			

R-Square	C.V.	Root MSE	B Mean
0.045124	41.34133	2.26931742	5.48922203

Source	DF	Anova SS	Mean Square	F Value	Pr > F
MF	1	24.09276868	24.09276868		

4.68 0.0330

Analysis of Variance Procedure

T tests (LSD) for variable: B

NOTE: This test controls the type I comparisonwise error rate not the experimentwise error rate.

Alpha= 0.05 df= 99 MSE= 5.149802
Critical Value of T= 1.98
Least Significant Difference= 0.9061
WARNING: Cell sizes are not equal.
Harmonic Mean of cell sizes= 49.38614

Means with the same letter are not significantly different.

T Grouping	Mean	N	MF
A	5.9098	58	0
B	4.9220	43	1

Analysis of Variance Procedure

Dependent Variable: TEMP

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	129205556.06701600	129205556.06701600	0.01	0.9107
Error	78	795994087326.48300000	10205052401.62150000		
Corrected Total	79	796123292882.55000000			

R-Square	C.V.	Root MSE	TEMP Mean
0.000162	-522.7881	101020.05940219	-19323.32858403

Source	DF	Anova SS	Mean Square	F Value	Pr > F

MF	1	129205556.06698200	129205556.06698200	0.01	0.9107

Analysis of Variance Procedure

Dependent Variable: LNTEMP

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	170116.34844989	170116.34844989	0.03	0.8663
Error	78	465196502.43647900	5964057.72354461		
Corrected Total	79	465366618.78492900			

R-Square	C.V.	Root MSE	LNTEMP Mean
0.000366	-553.3111	2442.14203591	-441.36869358

Source	DF	Anova SS	Mean Square	F Value	Pr > F
MF	1	170116.34844987	170116.34844987	0.03	0.8663

Analysis of Variance Procedure

T tests (LSD) for variable: TEMP

NOTE: This test controls the type I comparisonwise error rat
e not the
experimentwise error rate.

Alpha= 0.05 df= 78 MSE= 1.021E10
Critical Value of T= 1.99
Least Significant Difference= 47142
WARNING: Cell sizes are not equal.
Harmonic Mean of cell sizes= 36.4

Means with the same letter are not significantly different.

T Grouping	Mean	N	MF
A	-18391	52	0

A			
A	-21055	28	1

Analysis of Variance Procedure

T tests (LSD) for variable: LNTEMP

NOTE: This test controls the type I comparisonwise error rate not the experimentwise error rate.

Alpha= 0.05 df= 78 MSE= 5964058
Critical Value of T= 1.99
Least Significant Difference= 1139.7
WARNING: Cell sizes are not equal.
Harmonic Mean of cell sizes= 36.4

Means with the same letter are not significantly different.

T Grouping	Mean	N	MF
A	-378.5	28	1
A			
A	-475.2	52	0

Analysis of Variance Procedure
Class Level Information

Class	Levels	Values
GROUP	10	0 1 2 3 4 5 6 7 8 9
AI	2	0 1
MF	2	0 1
AGE20	5	0 1 2 3 4

Number of observations in data set = 130

Group	Obs	Dependent Variables
1	96	INVSL
2	127	AGE PA

3	115	HB
4	112	L
5	101	B
6	80	TEMP LNTEMP

NOTE: Variables in each group are consistent with respect to the presence or absence of missing values.

Analysis of Variance Procedure

Dependent Variable: INVSL

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	1.81387134	0.45346783	10.39	0.0001
Error	91	3.97294509	0.04365874		
Corrected Total	95	5.78681643			

R-Square	C.V.	Root MSE	INVSL Mean
0.313449	37.38120	0.20894673	0.55896207

Source	DF	Anova SS	Mean Square	F Value	Pr > F
AGE20	4	1.81387134	0.45346783	10.39	0.0001

Analysis of Variance Procedure

T tests (LSD) for variable: INVSL

NOTE: This test controls the type I comparisonwise error rat
e not the
experimentwise error rate.

Alpha= 0.05 Confidence= 0.95 df= 91 MSE= 0.043659
Critical Value of T= 1.98638

Comparisons significant at the 0.05 level are indicated by '***
'.

AGE20 Comparison		Lower Confidence Limit	Difference Between Means	Upper Confidence Limit	
0	- 1	-0.07806	0.04651	0.17107	
0	- 2	0.12340	0.25743	0.39146	***
0	- 3	0.16257	0.30294	0.44330	***
0	- 4	0.20962	0.39601	0.58240	***
1	- 0	-0.17107	-0.04651	0.07806	
1	- 2	0.09597	0.21092	0.32587	***
1	- 3	0.13414	0.25643	0.37871	***
1	- 4	0.17632	0.34950	0.52269	***
2	- 0	-0.39146	-0.25743	-0.12340	***
2	- 1	-0.32587	-0.21092	-0.09597	***
2	- 3	-0.08640	0.04551	0.17742	
2	- 4	-0.04153	0.13858	0.31869	
3	- 0	-0.44330	-0.30294	-0.16257	***
3	- 1	-0.37871	-0.25643	-0.13414	***
3	- 2	-0.17742	-0.04551	0.08640	
3	- 4	-0.09180	0.09308	0.27795	
4	- 0	-0.58240	-0.39601	-0.20962	***
4	- 1	-0.52269	-0.34950	-0.17632	***
4	- 2	-0.31869	-0.13858	0.04153	
4	- 3	-0.27795	-0.09308	0.09180	

Analysis of Variance Procedure

Dependent Variable: AGE

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	51000.90364604	12750.22591151	412.70	0.0001
Error	122	3769.11357746	30.89437359		
Corrected Total	126	54770.01722350			

R-Square	C.V.	Root MSE	AGE Mean
0.931183	12.76588	5.55827074	43.54006299

Source	DF	Anova SS	Mean Square	F Value	Pr > F
AGE20	4	51000.90364604	12750.22591151	412.70	0.0001

Analysis of Variance Procedure

Dependent Variable: PA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	103584453.22676200	25896113.30669070	2.06	0.0898
Error	122	1531826405.96552000	12555954.14725830		
Corrected Total	126	1635410859.19228000			

R-Square	C.V.	Root MSE	PA Mean
0.063338	123.9913	3543.43818166	2857.81181102

Source	DF	Anova SS	Mean Square	F Value	Pr > F
AGE20	4	103584453.22676200	25896113.30669070	2.06	0.0898

Analysis of Variance Procedure

T tests (LSD) for variable: AGE

NOTE: This test controls the type I comparisonwise error rat
e not the
experimentwise error rate.

Alpha= 0.05 Confidence= 0.95 df= 122 MSE= 30.89437
Critical Value of T= 1.97960

Comparisons significant at the 0.05 level are indicated by '***
'.

AGE20 Comparison		Lower Confidence Limit	Difference Between Means	Upper Confidence Limit	
4	- 3	10.055	14.781	19.508	***
4	- 2	29.964	34.531	39.097	***
4	- 1	45.967	50.475	54.983	***
4	- 0	64.714	69.489	74.264	***
3	- 4	-19.508	-14.781	-10.055	***
3	- 2	16.816	19.749	22.683	***
3	- 1	32.853	35.694	38.535	***
3	- 0	51.460	54.708	57.955	***
2	- 4	-39.097	-34.531	-29.964	***
2	- 3	-22.683	-19.749	-16.816	***
2	- 1	13.378	15.945	18.511	***
2	- 0	31.948	34.958	37.969	***
1	- 4	-54.983	-50.475	-45.967	***
1	- 3	-38.535	-35.694	-32.853	***
1	- 2	-18.511	-15.945	-13.378	***
1	- 0	16.093	19.014	21.934	***
0	- 4	-74.264	-69.489	-64.714	***
0	- 3	-57.955	-54.708	-51.460	***
0	- 2	-37.969	-34.958	-31.948	***
0	- 1	-21.934	-19.014		

Analysis of Variance Procedure

T tests (LSD) for variable: PA

NOTE: This test controls the type I comparisonwise error rat
e not the
experimentwise error rate.

Alpha= 0.05 Confidence= 0.95 df= 122 MSE= 12555954
Critical Value of T= 1.97960

Comparisons significant at the 0.05 level are indicated by '***

AGE20 Comparison		Lower Confidence Limit	Difference Between Means	Upper Confidence Limit	
0	- 3	-383.6	1686.9	3757.3	
0	- 1	-62.6	1799.3	3661.2	
0	- 2	526.6	2445.9	4365.2	***
0	- 4	379.7	3423.7	6467.6	***
3	- 0	-3757.3	-1686.9	383.6	
3	- 1	-1698.8	112.4	1923.6	
3	- 2	-1111.1	759.0	2629.1	
3	- 4	-1276.4	1736.8	4750.0	
1	- 0	-3661.2	-1799.3	62.6	
1	- 3	-1923.6	-112.4	1698.8	
1	- 2	-989.6	646.6	2282.9	
1	- 4	-1249.5	1624.4	4498.3	
2	- 0	-4365.2	-2445.9	-526.6	***
2	- 3	-2629.1	-759.0	1111.1	
2	- 1	-2282.9	-646.6	989.6	
2	- 4	-1933.7	977.8	3889.2	
4	- 0	-6467.6	-3423.7	-379.7	***
4	- 3	-4750.0	-1736.8	1276.4	
4	- 1	-4498.3	-1624.4	1249.5	
4	- 2	-3889.2	-977.8	1933.7	

Analysis of Variance Procedure

Dependent Variable: HB

Source	DF	Sum of Squares	Mean Square

	F Value	Pr > F		
Model		4	36.07530646	9.01882661
	1.73	0.1479		
Error		110	572.62366181	5.20566965
Corrected Total		114	608.69896826	

R-Square	C.V.	Root MSE	HB Mean
0.059266	14.80346	2.28159367	15.41256522

Source	DF	Anova SS	Mean Square	F Value	Pr > F
AGE20	4	36.07530646	9.01882661	1.73	0.1479

Analysis of Variance Procedure

T tests (LSD) for variable: HB

NOTE: This test controls the type I comparisonwise error rate not the experimentwise error rate.

Alpha= 0.05 Confidence= 0.95 df= 110 MSE= 5.20567
Critical Value of T= 1.98177

Comparisons significant at the 0.05 level are indicated by '***

AGE20 Comparison		Lower Confidence Limit	Difference Between Means	Upper Confidence Limit	
3	- 2	-0.7394	0.4966	1.7327	
3	- 1	0.0177	1.2314	2.4451	***
3	- 4	-0.7170	1.2348	3.1867	
3	- 0	0.1483	1.5712	2.9941	***
2	- 3	-1.7327	-0.4966	0.7394	
2	- 1	-0.3711	0.7348	1.8407	
2	- 4	-1.1485	0.7382	2.6249	
2	- 0	-0.2576	1.0746	2.4068	
1	- 3	-2.4451	-1.2314	-0.0177	***
1	- 2	-1.8407	-0.7348	0.3711	
1	- 4	-1.8687	0.0034	1.8755	
1	- 0	-0.9717	0.3398	1.6513	
4	- 3	-3.1867	-1.2348	0.7170	
4	- 2	-2.6249	-0.7382	1.1485	
4	- 1	-1.8755	-0.0034	1.8687	
4	- 0	-1.6777	0.3364	2.3504	
0	- 3	-2.9941	-1.5712	-0.1483	***
0	- 2	-2.4068	-1.0746	0.2576	
0	- 1	-1.6513	-0.3398	0.9717	
0	- 4	-2.3504	-0.3364	1.6777	

Analysis of Variance Procedure

Dependent Variable: L

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	108.74765946	27.18691486	4.41	0.0024
Error	107	659.77203911	6.16609382		
Corrected Total	111	768.51969857			

R-Square	C.V.	Root MSE	L Mean
0.141503	38.90647	2.48316206	6.38238777

Source	DF	Anova SS	Mean Square	F Value	Pr > F
AGE20	4	108.74765946	27.18691486	4.41	0.0024

Analysis of Variance Procedure

T tests (LSD) for variable: L

NOTE: This test controls the type I comparisonwise error rat
e not the
experimentwise error rate.

Alpha= 0.05 Confidence= 0.95 df= 107 MSE= 6.166094
Critical Value of T= 1.98238

Comparisons significant at the 0.05 level are indicated by '***

AGE20 Comparison		Lower Confidence Limit	Difference Between Means	Upper Confidence Limit	
0	- 1	-0.7450	0.7808	2.3067	
0	- 2	0.4039	1.9368	3.4697	***
0	- 3	0.5788	2.2125	3.8462	***
0	- 4	1.6205	3.8737	6.1270	***
1	- 0	-2.3067	-0.7808	0.7450	
1	- 2	-0.0469	1.1560	2.3589	
1	- 3	0.1027	1.4317	2.7607	***
1	- 4	1.0498	3.0929	5.1360	***
2	- 0	-3.4697	-1.9368	-0.4039	***
2	- 1	-2.3589	-1.1560	0.0469	
2	- 3	-1.0614	0.2757	1.6128	
2	- 4	-0.1115	1.9369	3.9853	
3	- 0	-3.8462	-2.2125	-0.5788	***
3	- 1	-2.7607	-1.4317	-0.1027	***
3	- 2	-1.6128	-0.2757	1.0614	
3	- 4	-0.4637	1.6612	3.7861	
4	- 0	-6.1270	-3.8737	-1.6205	***
4	- 1	-5.1360	-3.0929	-1.0498	***
4	- 2	-3.9853	-1.9369	0.1115	
4	- 3	-3.7861	-1.6612	0.4637	

Analysis of Variance Procedure

Dependent Variable: B

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	76.79301733	19.19825433	4.03	0.0046
Error	96	457.13010582	4.76177194		
Corrected Total	100	533.92312315			

R-Square	C.V.	Root MSE	B Mean
0.143828	39.75333	2.18214847	5.48922203

Source	DF	Anova SS	Mean Square	F Value	Pr > F
AGE20	4	76.79301733	19.19825433	4.03	0.0046

Analysis of Variance Procedure

T tests (LSD) for variable: B

NOTE: This test controls the type I comparisonwise error rate not the experimentwise error rate.

Alpha= 0.05 Confidence= 0.95 df= 96 MSE= 4.761772
Critical Value of T= 1.98498

Comparisons significant at the 0.05 level are indicated by '***'

AGE20 Comparison	Lower Confidence Limit	Difference Between Means	Upper Confidence Limit	
0 - 1	-0.7182	0.6227	1.9636	
0 - 2	0.1963	1.5537	2.9112	***
0 - 3	0.1742	1.6116	3.0489	***
0 - 4	1.6026	3.6762	5.7498	***

1	- 0	-1.9636	-0.6227	0.7182	
1	- 2	-0.2071	0.9311	2.0692	
1	- 3	-0.2435	0.9889	2.2213	
1	- 4	1.1164	3.0535	4.9907	***
2	- 0	-2.9112	-1.5537	-0.1963	***
2	- 1	-2.0692	-0.9311	0.2071	
2	- 3	-1.1926	0.0578	1.3082	
2	- 4	0.1739	2.1225	4.0711	***
3	- 0	-3.0489	-1.6116	-0.1742	***
3	- 1	-2.2213	-0.9889	0.2435	
3	- 2	-1.3082	-0.0578	1.1926	
3	- 4	0.0595	2.0646	4.0698	***
4	- 0	-5.7498	-3.6762	-1.6026	***
4	- 1	-4.9907	-3.0535	-1.1164	***
4	- 2	-4.0711	-2.1225	-0.1739	***
4	- 3	-4.0698	-2.0646	-0.0595	***

Analysis of Variance Procedure

Dependent Variable: TEMP

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	14291397493.12870000	3572849373.28219000	0.34	0.8483
Error	75	781831895389.42100000	10424425271.85890000		
Corrected Total	79	796123292882.55000000			

R-Square	C.V.	Root MSE	TEMP Mean
0.017951	-528.3773	102100.07478870	-19323.32858403

Source	DF	Anova SS	Mean Square	F Value	Pr > F
AGE20	4	14291397493.12870000	3572849373.28219000	0.34	0.8483

Analysis of Variance Procedure

Dependent Variable: LNTEMP

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	15705220.88035810	3926305.22008953	0.65	0.6253
Error	75	449661397.90457100	5995485.30539429		
Corrected Total	79	465366618.78492900			

R-Square	C.V.	Root MSE	LNTEMP Mean
0.033748	-554.7670	2448.56801118	-441.36869358

Source	DF	Anova SS	Mean Square	F Value	Pr > F
AGE20	4	15705220.88035810	3926305.22008953	0.65	0.6253

Analysis of Variance Procedure

T tests (LSD) for variable: TEMP

NOTE: This test controls the type I comparisonwise error rat
e not the
experimentwise error rate.

Alpha= 0.05 Confidence= 0.95 df= 75 MSE= 1.042E10
Critical Value of T= 1.99210

Comparisons significant at the 0.05 level are indicated by '***
'.

AGE20 Comparison		Lower Confidence Limit	Difference Between Means	Upper Confidence Limit
3	- 1	-63024	1375	65775
3	- 0	-55990	13024	82037
3	- 2	-46924	19066	85055
3	- 4	-49401	52830	155061
1	- 3	-65775	-1375	63024
1	- 0	-55846	11648	79143
1	- 2	-46709	17691	82090
1	- 4	-49757	51455	152666
0	- 3	-82037	-13024	55990
0	- 1	-79143	-11648	55846
0	- 2	-62971	6042	75056
0	- 4	-64402	39806	144015
2	- 3	-85055	-19066	46924
2	- 1	-82090	-17691	46709
2	- 0	-75056	-6042	62971
2	- 4	-68467	33764	135995
4	- 3	-155061	-52830	49401
4	- 1	-152666	-51455	49757
4	- 0	-144015	-39806	64402
4	- 2	-135995	-33764	68467

Analysis of Variance Procedure

T tests (LSD) for variable: LNTEMP

NOTE: This test controls the type I comparisonwise error rat
e not the
experimentwise error rate.

Alpha= 0.05 Confidence= 0.95 df= 75 MSE= 5995485
Critical Value of T= 1.99210

Comparisons significant at the 0.05 level are indicated by '***
'.

AGE20 Comparison		Lower Confidence Limit	Difference Between Means	Upper Confidence Limit
3	- 1	-1391.2	153.3	1697.7
3	- 0	-1412.9	242.2	1897.3
3	- 2	-901.8	680.7	2263.3
3	- 4	-665.6	1786.1	4237.8
1	- 3	-1697.7	-153.3	1391.2
1	- 0	-1529.7	88.9	1707.6
1	- 2	-1017.0	527.5	2071.9
1	- 4	-794.4	1632.9	4060.1
0	- 3	-1897.3	-242.2	1412.9
0	- 1	-1707.6	-88.9	1529.7
0	- 2	-1216.6	438.5	2093.6
0	- 4	-955.2	1543.9	4043.0
2	- 3	-2263.3	-680.7	901.8
2	- 1	-2071.9	-527.5	1017.0
2	- 0	-2093.6	-438.5	1216.6
2	- 4	-1346.3	1105.4	3557.1
4	- 3	-4237.8	-1786.1	665.6
4	- 1	-4060.1	-1632.9	794.4
4	- 0	-4043.0	-1543.9	955.2
4	- 2	-3557.1	-1105.4	1346.3

Appendix K

General Linear Models Procedure

Number of observations in data set = 129

NOTE: Due to missing values, only 61 observations can be used in this analysis.

General Linear Models Procedure

Dependent Variable: INVSLOPE

Source	DF	Sum of Squares	F Value	Pr > F
Model	7	2.98608161	31.20	0.0001
Error	53	0.72462699		
Corrected Total	60	3.71070860		

R-Square	C.V.	INVSLOPE Mean
0.804720	19.87374	0.58835535

Source	DF	Type I SS	F Value	Pr > F
AGE	1	1.56193659	114.24	0.0001
HB	1	0.00014242	0.01	0.9191
PA	1	0.39310065	28.75	0.0001
L	1	0.94411729	69.05	0.0001
B	1	0.02029400	1.48	0.2285
TEMP	1	0.01947648	1.42	0.2380
LNTEMP	1	0.04701417	3.44	0.0693

Source	DF	Type III SS	F Value	Pr > F
AGE	1	0.06140282	4.49	0.0388
HB	1	0.00004202	0.00	0.9560
PA	1	0.16529319	12.09	0.0010
L	1	0.04562573	3.34	0.0734
B	1	0.01842426	1.35	0.2509
TEMP	1	0.05170827	3.78	0.0571
LNTEMP	1	0.04701417	3.44	0.0693

Parameter	Estimate	T for H0: Parameter=0	Pr > |T|	Std Error of Estimate
INTERCEPT	0.2141028498	1.90	0.0628	0.11264168
AGE	-.0018842877	-2.12	0.0388	0.00088914
HB	0.0003277931	0.06	0.9560	0.00591294
PA	0.0000139304	3.48	0.0010	0.00000401

L	0.0405076592	1.83	0.0734	0.02217438
B	0.0259043296	1.16	0.2509	0.02231499
TEMP	-.0000033854	-1.94	0.0571	0.00000174
LNTEMP	0.0001333922	1.85	0.0693	0.00007193

General Linear Models Procedure

Number of observations in data set = 129

NOTE: Due to missing values, only 94 observations can be used in this analysis.

General Linear Models Procedure

Dependent Variable: INVSLOPE

Source	DF	Sum of Squares	F Value	Pr > F
Model	1	3.91385206	192.78	0.0001
Error	92	1.86778567		
Corrected Total	93	5.78163774		

R-Square	C.V.	INVSLOPE Mean
0.676945	25.53721	0.55795118

Source	DF	Type I SS	F Value	Pr > F
L	1	3.91385206	192.78	0.0001

Source	DF	Type III SS	F Value	Pr > F
L	1	3.91385206	192.78	0.0001

Parameter	Estimate	T for H0: Parameter=0	Pr > |T|	Std Error of Estimate
INTERCEPT	0.0550493927	1.41	0.1624	0.03908811
L	0.0845292547	13.88	0.0001	0.00608800

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Correlation Analysis

8 'VAR' Variables: INVSLOPE AGE HB PA L B TEMP LNTEMP

Simple Statistics

Variable	N	Mean	Std Dev	Sum	Minimum	Maximum
INVSLOPE	96	0.5590	0.2468	53.6604	0.1458	1.2851
AGE	129	43.2371	20.8270	5577.6	7.0000	86.8300
HB	117	15.3792	2.3055	1799.4	10.1250	24.8250
PA	127	2857.8	3602.7	362942	0	24584.0
L	114	6.3121	2.6725	719.6	0.5010	12.4632
B	102	5.4566	2.3227	556.6	1.8420	10.4750
TEMP	80	-19323.3	100387	-1545866	-783221	188363
LNTEMP	80	-441.4	2427.1	-35309.6	-19117.4	3822.8

Correlation Analysis

Pearson Correlation Coefficients / Prob > |R| under Ho: Rho=0 / Number of Observations

	INVSLOPE	AGE	HB	PA
INVSLOPE	1.00000 0.0 96	-0.57573 0.0001 96	-0.11318 0.2722 96	0.40022 0.0001 96
AGE	-0.57573 0.0001 96	1.00000 0.0 129	0.20543 0.0263 117	-0.21137 0.0171 127
HB	-0.11318 0.2722 96	0.20543 0.0263 117	1.00000 0.0 117	-0.11806 0.2089 115
PA	0.40022 0.0001 96	-0.21137 0.0171 127	-0.11806 0.2089 115	1.00000 0.0 127
L	0.82277 0.0001 94	-0.32625 0.0004 114	-0.05156 0.5876 113	0.20354 0.0314 112
B	0.80561 0.0001 87	-0.32133 0.0010 102	-0.03469 0.7292 102	0.19643 0.0490 101
TEMP	-0.01529 0.9015 68	-0.00537 0.9623 80	0.03552 0.7544 80	0.11341 0.3165 80

LNTEMP	0.03124	-0.03738	0.02882	0.11850
	0.8003	0.7420	0.7997	0.2951
	68	80	80	80

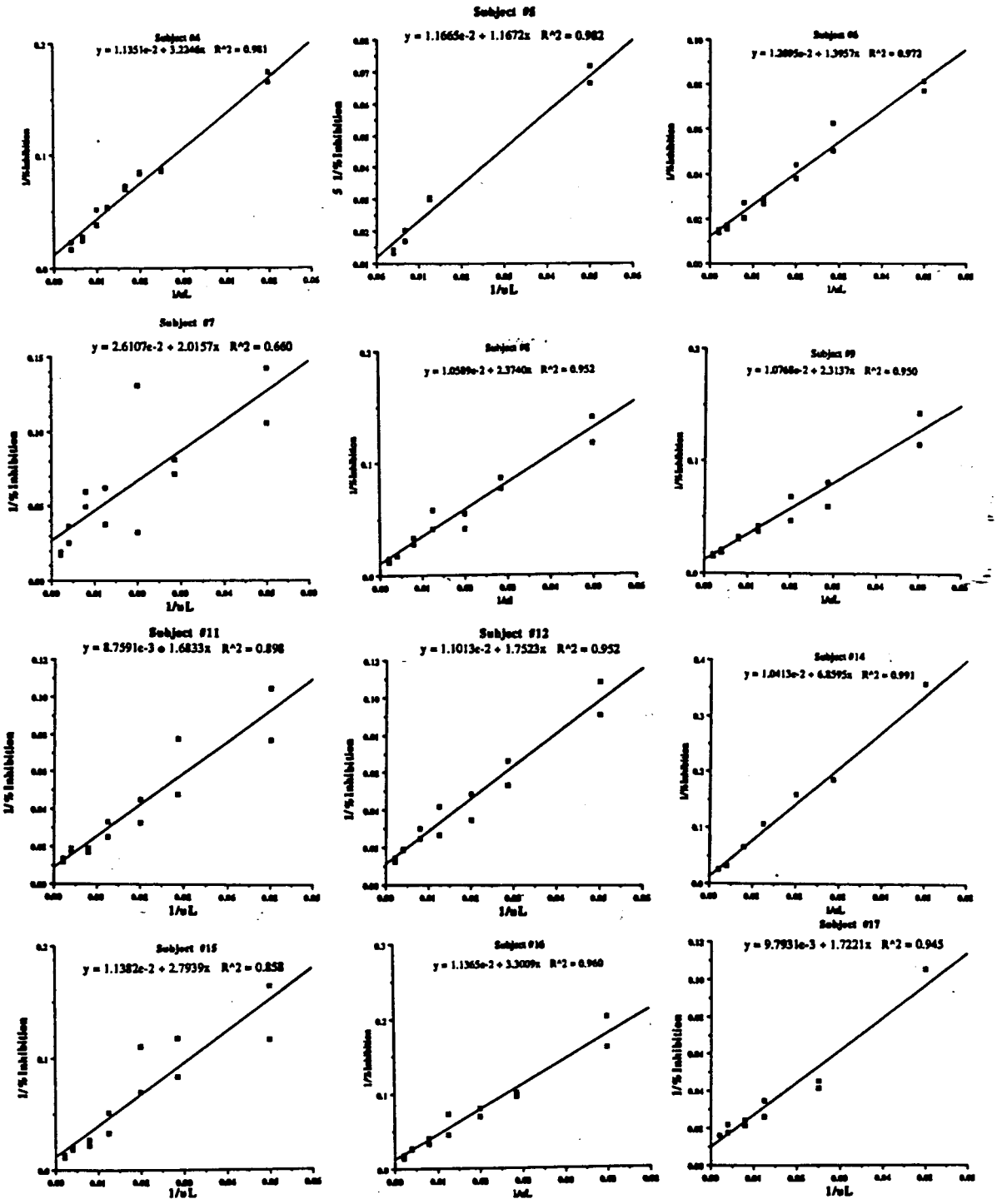
Correlation Analysis

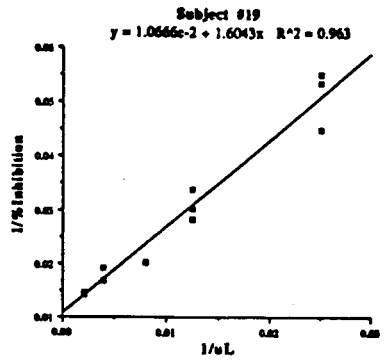
Pearson Correlation Coefficients / Prob > |R| under Ho: Rho=0
/ Number of Observations

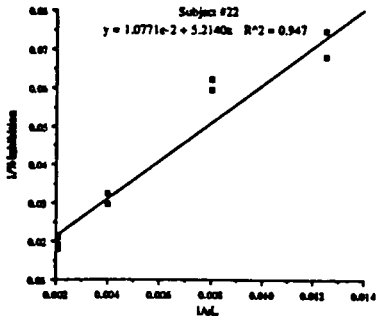
	L	B	TEMP	LNTEMP
INVSLOPE	0.82277	0.80561	-0.01529	0.03124
	0.0001	0.0001	0.9015	0.8003
	94	87	68	68
AGE	-0.32625	-0.32133	-0.00537	-0.03738
	0.0004	0.0010	0.9623	0.7420
	114	102	80	80
HB	-0.05156	-0.03469	0.03552	0.02882
	0.5876	0.7292	0.7544	0.7997
	113	102	80	80
PA	0.20354	0.19643	0.11341	0.11850
	0.0314	0.0490	0.3165	0.2951
	112	101	80	80
L	1.00000	0.96176	0.03129	0.02646
	0.0	0.0001	0.7842	0.8169
	114	100	79	79
B	0.96176	1.00000	0.09026	0.07223
	0.0001	0.0	0.4574	0.5524
	100	102	70	70
TEMP	0.03129	0.09026	1.00000	0.98476
	0.7842	0.4574	0.0	0.0001
	79	70	80	80
LNTEMP	0.02646	0.07223	0.98476	1.00000
	0.8169	0.5524	0.0001	0.0
	79	70	80	80

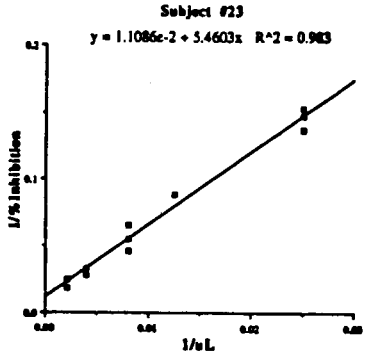
Appendix L

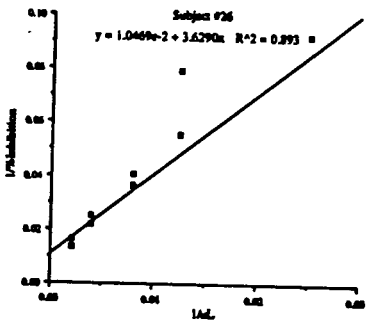
Collection of 1/%Inhibition vs 1/uL SOD plots for all subjects

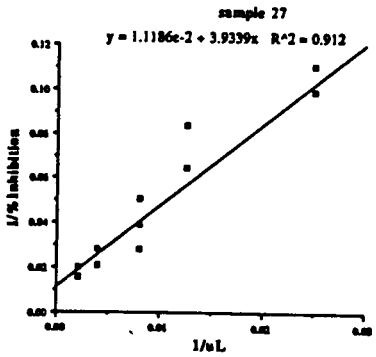


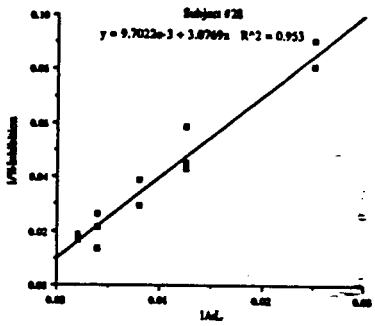

Subject #19
y = 1.0666e-2 + 1.6043x R^2 = 0.963
1/% Inhibition
1/uL

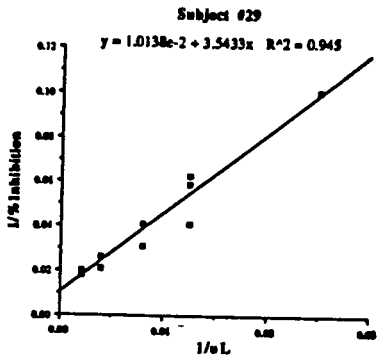

Subject #22
y = 1.0771e-2 + 5.2140x R^2 = 0.947
1/% Inhibition
1/uL

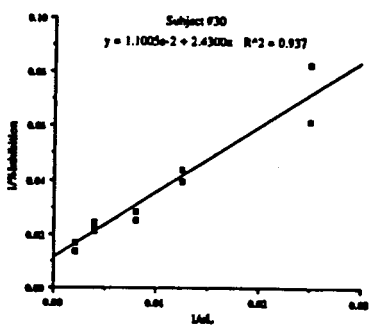

Subject #23
y = 1.1086e-2 + 5.4603x R^2 = 0.983
1/% Inhibition
1/uL

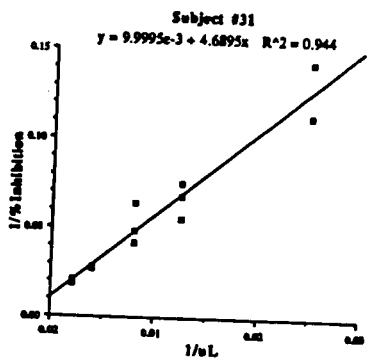

Subject #26
y = 1.0469e-2 + 3.6290x R^2 = 0.893
1/% Inhibition
1/uL

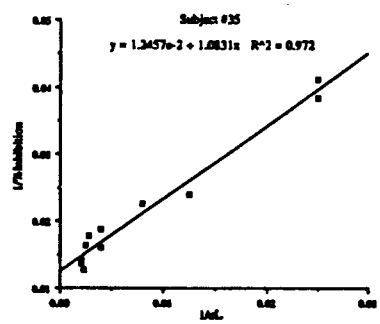

sample 27
y = 1.1186e-2 + 3.9339x R^2 = 0.912
1/% Inhibition
1/uL

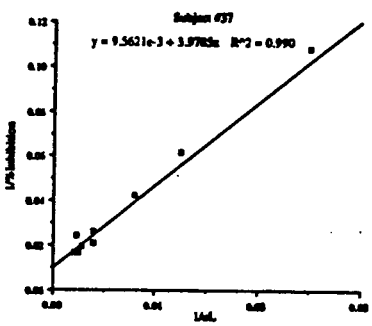

Subject #28
y = 9.7022e-3 + 3.8769x R^2 = 0.953
1/% Inhibition
1/uL

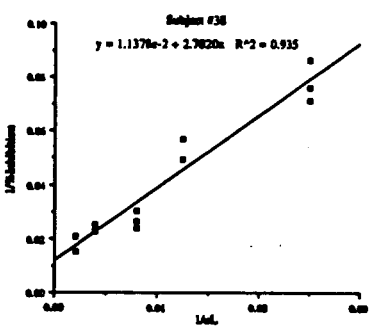

Subject #29
y = 1.0138e-2 + 3.5433x R^2 = 0.945
1/% Inhibition
1/uL

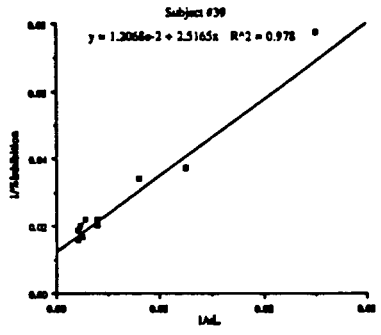

Subject #30
y = 1.1005e-2 + 2.4300x R^2 = 0.937
1/% Inhibition
1/uL

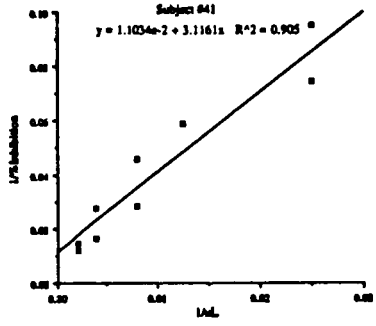

Subject #31
y = 9.9995e-3 + 4.6895x R^2 = 0.944
1/% Inhibition
1/uL

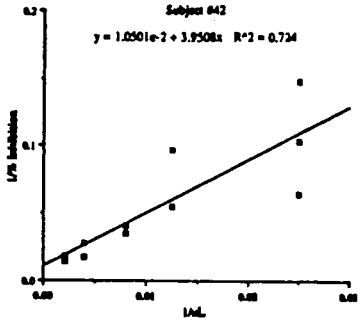

Subject #35
y = 1.3457e-2 + 1.0831x R^2 = 0.972
1/% Inhibition
1/uL

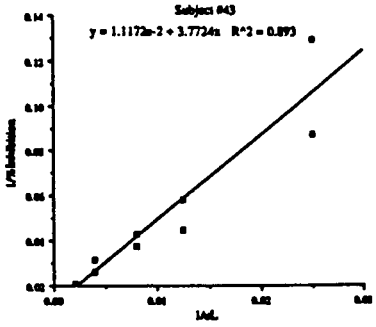

Subject #37
y = 9.5621e-3 + 3.9785x R^2 = 0.990
1/% Inhibition
1/uL

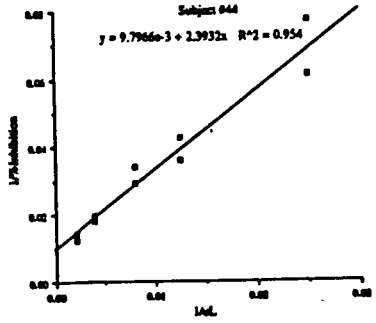

Subject #38
y = 1.1378e-2 + 2.7820x R^2 = 0.935
1/% Inhibition
1/uL

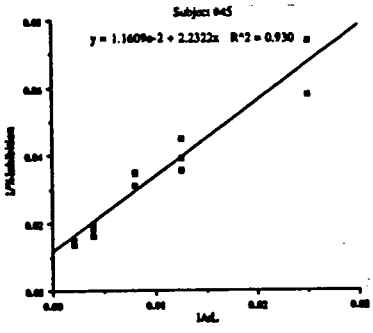

Subject #39
y = 1.2068e-2 + 2.5165x R^2 = 0.978
1/%Inhibition
1/Aol.

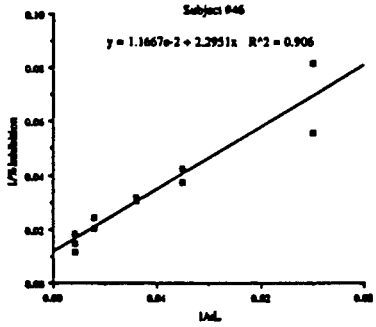

Subject #41
y = 1.1034e-2 + 3.1161x R^2 = 0.905
1/%Inhibition
1/Aol.

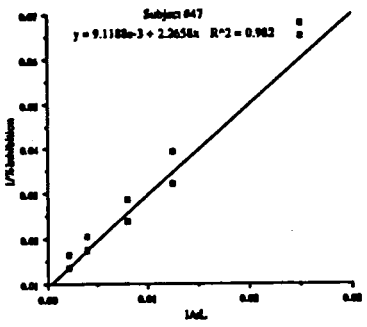

Subject #42
y = 1.0501e-2 + 3.9508x R^2 = 0.734
1/%Inhibition
1/Aol.

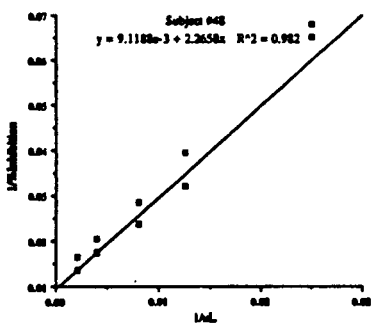

Subject #43
y = 1.1172e-2 + 3.7724x R^2 = 0.893
1/%Inhibition
1/Aol.

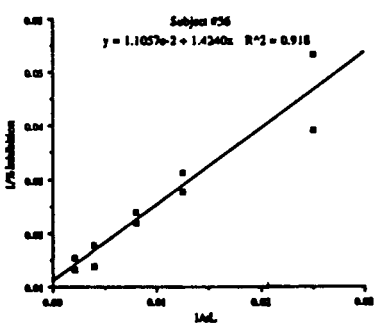

Subject #44
y = 9.7966e-3 + 2.3932x R^2 = 0.954
1/%Inhibition
1/Aol.

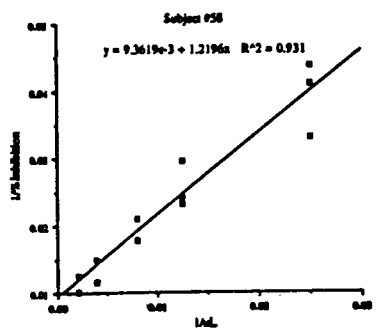

Subject #45
y = 1.1609e-2 + 2.2322x R^2 = 0.930
1/%Inhibition
1/Aol.

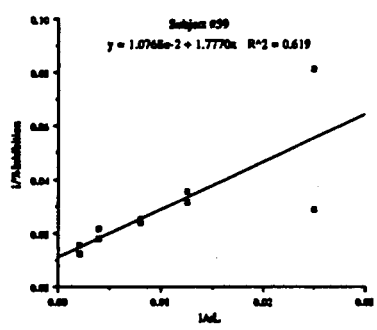

Subject #46
y = 1.1667e-2 + 2.2951x R^2 = 0.908
1/%Inhibition
1/Aol.

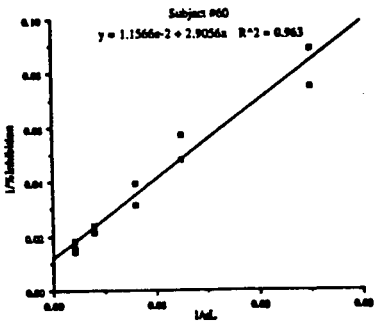

Subject #47
y = 9.1188e-3 + 2.2658x R^2 = 0.982
1/%Inhibition
1/Aol.

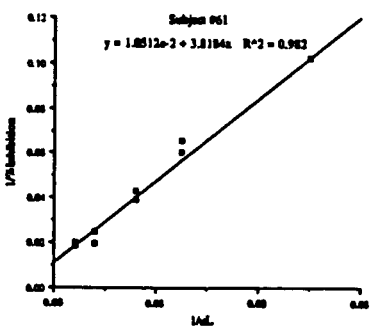

Subject #48
y = 9.1188e-3 + 2.2658x R^2 = 0.982
1/%Inhibition
1/Aol.

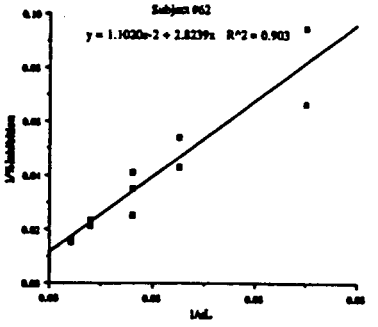

Subject #56
y = 1.1057e-2 + 1.4240x R^2 = 0.918
1/%Inhibition
1/Aol.

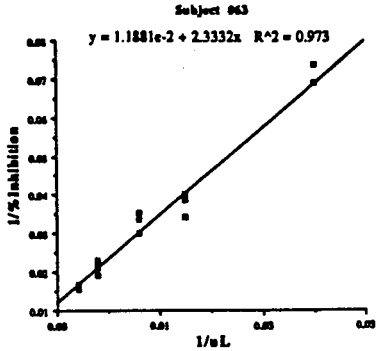

Subject #58
y = 9.3619e-3 + 1.2196x R^2 = 0.931
1/%Inhibition
1/Aol.

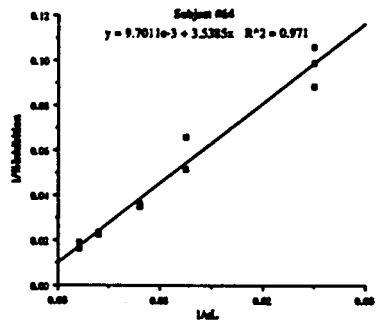

Subject #59
y = 1.0768e-2 + 1.7770x R^2 = 0.619
1/%Inhibition
1/Aol.

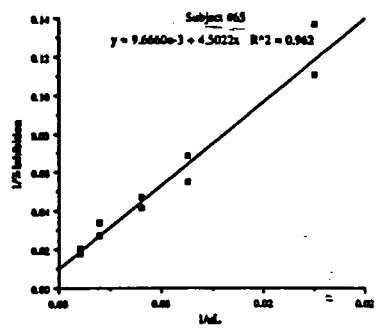

Subject #60
y = 1.1566e-2 + 2.9056x R^2 = 0.963
1/%Inhibition
1/uL

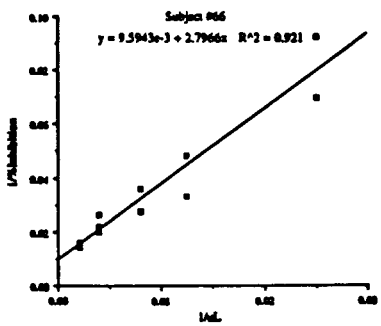

Subject #61
y = 1.8512e-2 + 3.8184x R^2 = 0.982
1/%Inhibition
1/uL

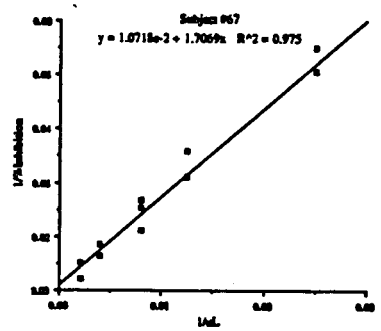

Subject #62
y = 1.1020e-2 + 2.8239x R^2 = 0.903
1/%Inhibition
1/uL

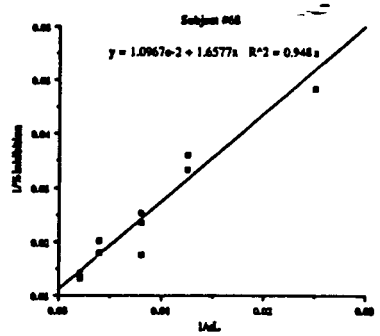

Subject #63
y = 1.1881e-2 + 2.3332x R^2 = 0.973
1/%Inhibition
1/uL

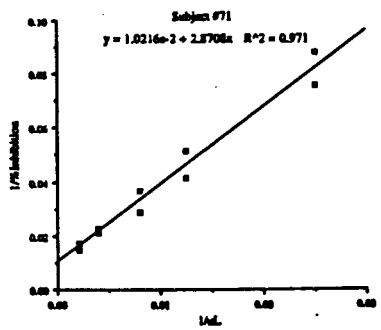

Subject #64
y = 9.7011e-3 + 3.5385x R^2 = 0.971
1/%Inhibition
1/uL

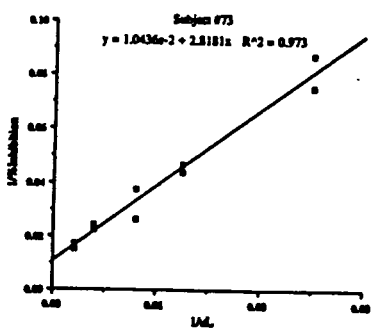

Subject #65
y = 9.6660e-3 + 4.5022x R^2 = 0.962
1/%Inhibition
1/uL

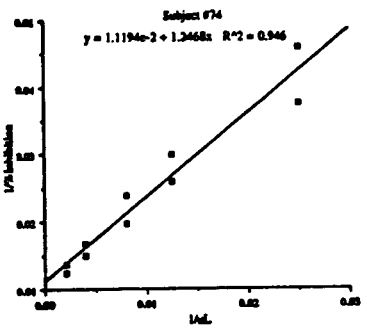

Subject #66
y = 9.5943e-3 + 2.7966x R^2 = 0.921
1/%Inhibition
1/uL

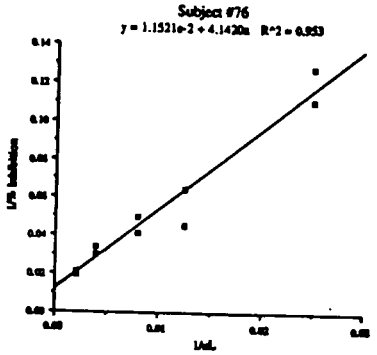

Subject #67
y = 1.0718e-2 + 1.7069x R^2 = 0.975
1/%Inhibition
1/uL

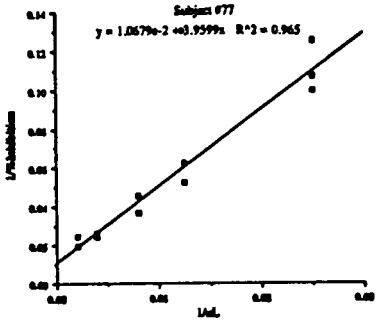

Subject #68
y = 1.0967e-2 + 1.6577x R^2 = 0.948
1/%Inhibition
1/uL

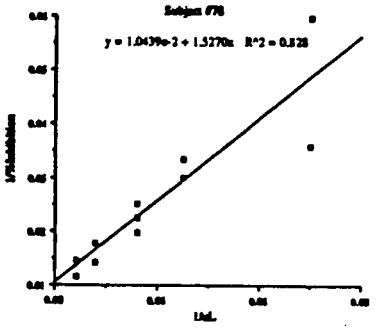

Subject #71
y = 1.0216e-2 + 2.8708x R^2 = 0.971
1/%Inhibition
1/uL

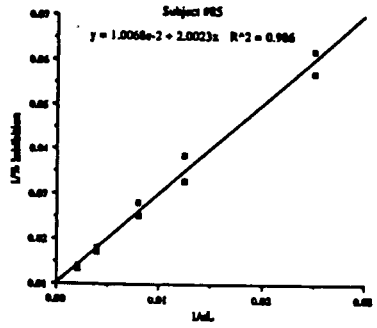

Subject #73
y = 1.0436e-2 + 2.8181x R^2 = 0.973
1/%Inhibition
1/uL

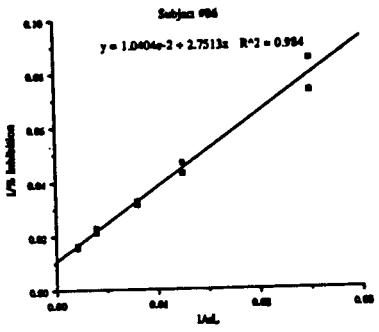

Subject #74
y = 1.1194e-2 + 1.3468x R^2 = 0.946
1/%Inhibition
1/uL

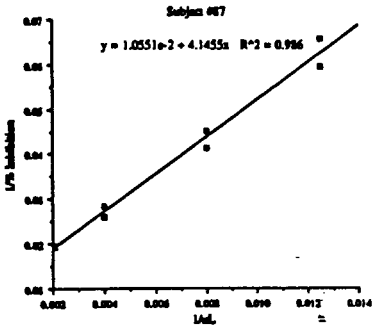

Subject #76
y = 1.1521e-2 + 4.1420x R^2 = 0.953
1/% Inhibition
1/Aβ1-40

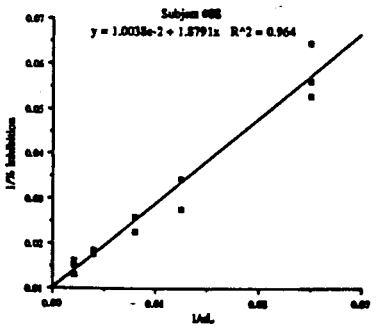

Subject #77
y = 1.0679e-2 +e3.9599x R^2 = 0.965
1/% Inhibition
1/Aβ1-40

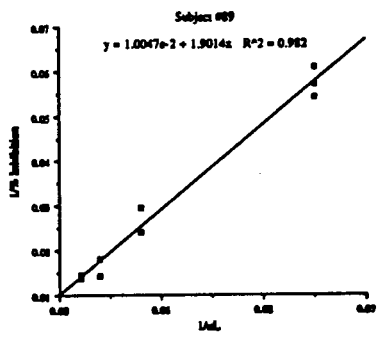

Subject #78
y = 1.0439e-2 + 1.5270x R^2 = 0.828
1/% Inhibition
1/Aβ1-40

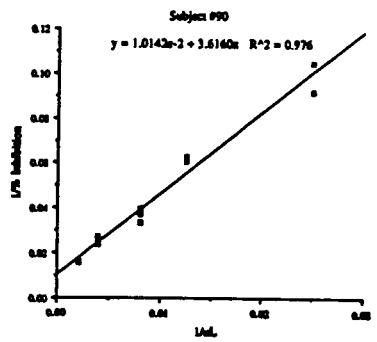

Subject #85
y = 1.0068e-2 + 2.0023x R^2 = 0.986
1/% Inhibition
1/Aβ1-40

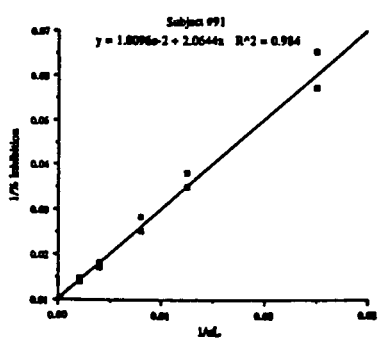

Subject #86
y = 1.0404e-2 + 2.7513x R^2 = 0.984
1/% Inhibition
1/Aβ1-40

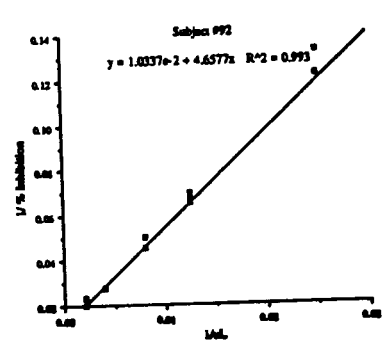

Subject #87
y = 1.0551e-2 + 4.1455x R^2 = 0.986
1/% Inhibition
1/Aβ1-40

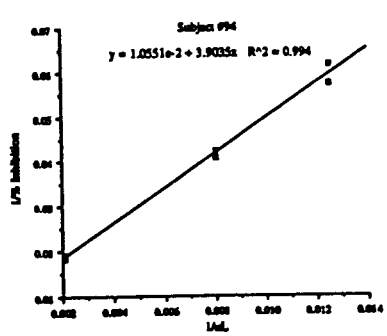

Subject #88
y = 1.0038e-2 + 1.8791x R^2 = 0.964
1/% Inhibition
1/Aβ1-40

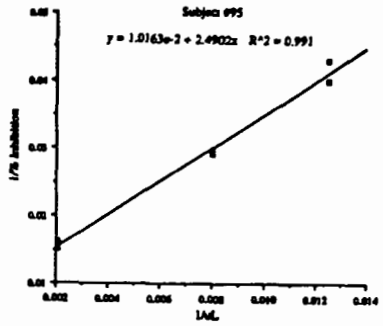

Subject #89
y = 1.0047e-2 + 1.9014x R^2 = 0.982
1/% Inhibition
1/Aβ1-40

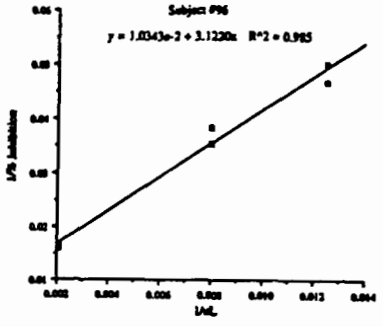

Subject #90
y = 1.0142e-2 + 3.6160x R^2 = 0.976
1/% Inhibition
1/Aβ1-40

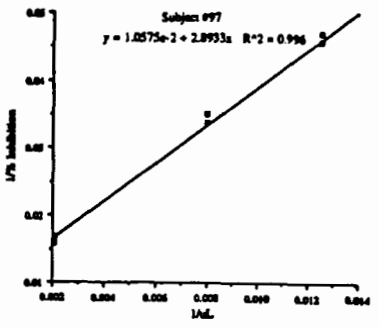

Subject #91
y = 1.8096e-2 + 2.0644x R^2 = 0.984
1/% Inhibition
1/Aβ1-40

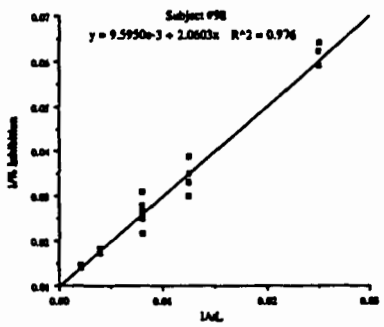

Subject #92
y = 1.0337e-2 + 4.6577x R^2 = 0.993
1/% Inhibition
1/Aβ1-40

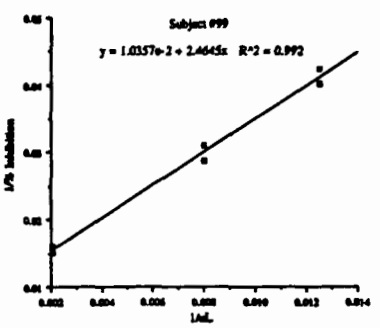

Subject #94
y = 1.0551e-2 + 3.9035x R^2 = 0.994
1/% Inhibition
1/Aβ1-40

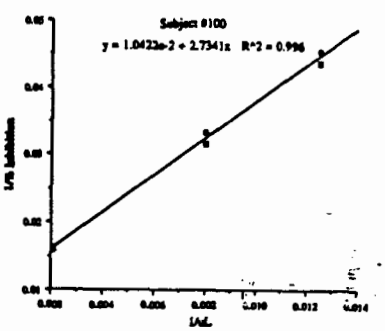

Subject #95
y = 1.0163e-2 + 2.4902x R^2 = 0.991
1/% Inhibition
1/Aℓ.

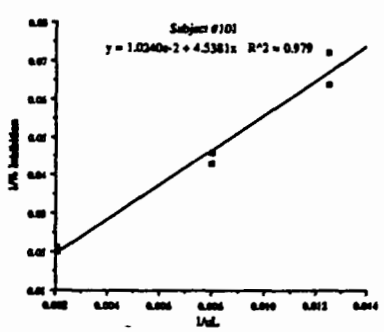

Subject #96
y = 1.0343e-2 + 3.1220x R^2 = 0.985
1/% Inhibition
1/Aℓ.

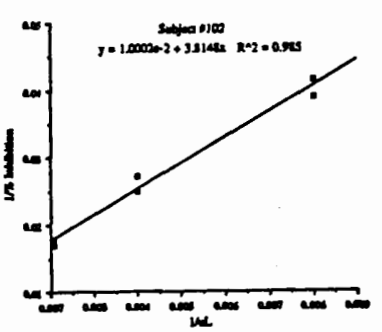

Subject #97
y = 1.0575e-2 + 2.8953x R^2 = 0.996
1/% Inhibition
1/Aℓ.

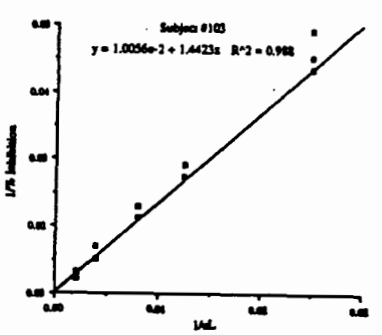

Subject #98
y = 9.5950e-3 + 2.0603x R^2 = 0.976
1/% Inhibition
1/Aℓ.

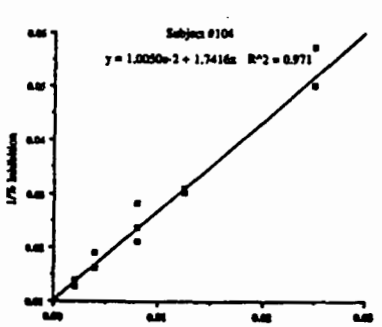

Subject #99
y = 1.0357e-2 + 2.4645x R^2 = 0.992
1/% Inhibition
1/Aℓ.

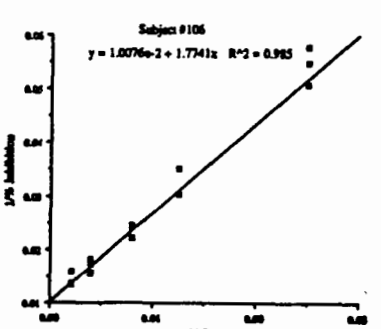

Subject #100
y = 1.0423e-2 + 2.7341x R^2 = 0.996
1/% Inhibition
1/Aℓ.

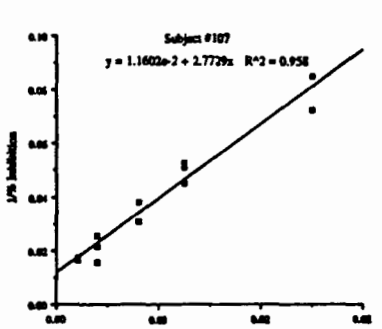

Subject #101
y = 1.0240e-2 + 4.5381x R^2 = 0.979
1/% Inhibition
1/Aℓ.

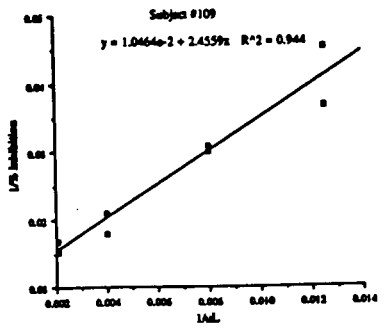

Subject #102
y = 1.0003e-2 + 3.8148x R^2 = 0.985
1/% Inhibition
1/Aℓ.

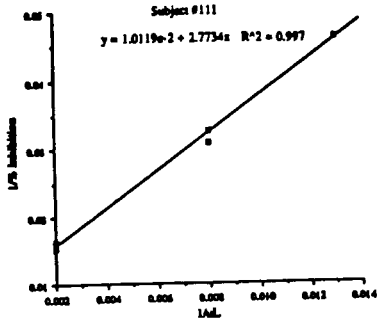

Subject #103
y = 1.0056e-2 + 1.4423x R^2 = 0.988
1/% Inhibition
1/Aℓ.

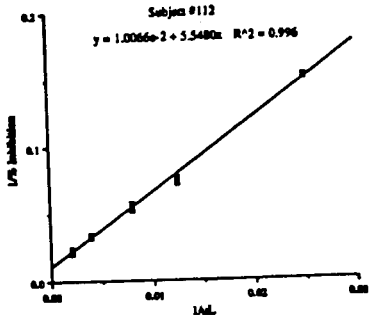

Subject #104
y = 1.0050e-2 + 1.7416x R^2 = 0.971
1/% Inhibition
1/Aℓ.

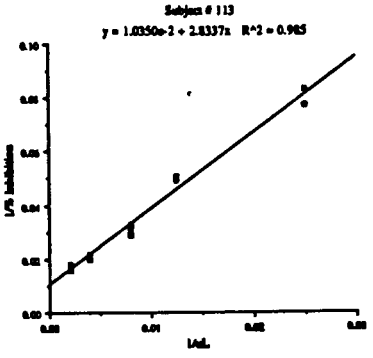

Subject #106
y = 1.0076e-2 + 1.7741x R^2 = 0.985
1/% Inhibition
1/Aℓ.

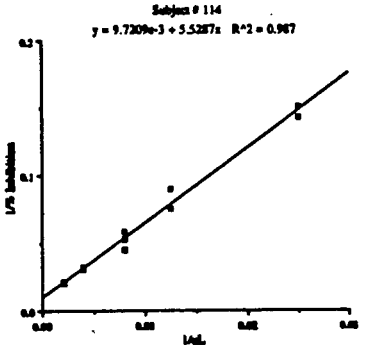

Subject #107
y = 1.1602e-2 + 2.7729x R^2 = 0.958
1/% Inhibition
1/Aℓ.

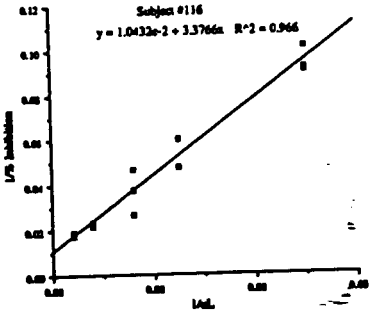

Subject #109
y = 1.0464e-2 + 2.4559x R^2 = 0.944
1/% Inhibition
1/nL

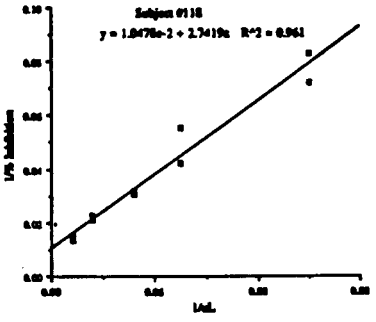

Subject #111
y = 1.0119e-2 + 2.7734x R^2 = 0.997
1/% Inhibition
1/nL

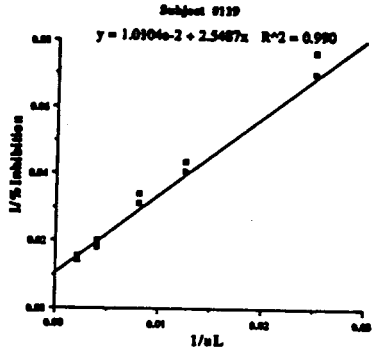

Subject #112
y = 1.0066e-2 + 5.5480x R^2 = 0.996
1/% Inhibition
1/nL

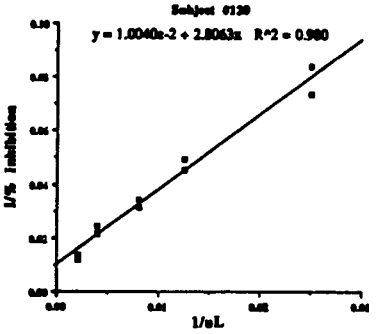

Subject # 113
y = 1.0350e-2 + 2.8337x R^2 = 0.985
1/% Inhibition
1/nL

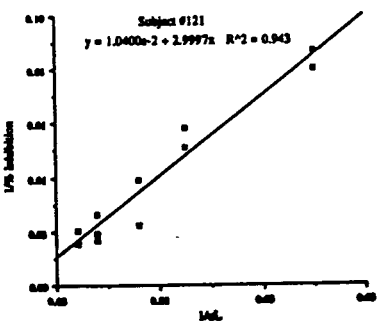

Subject # 114
y = 9.7209e-3 + 5.5287x R^2 = 0.987
1/% Inhibition
1/nL

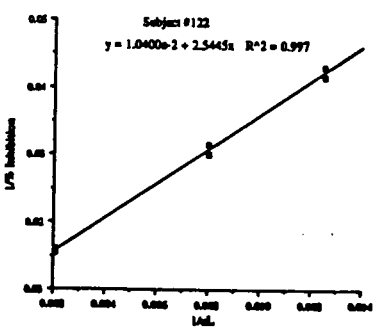

Subject #116
y = 1.0432e-2 + 3.3766x R^2 = 0.966
1/% Inhibition
1/nL

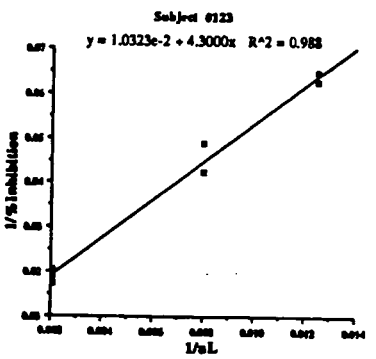

Subject #118
y = 1.0478e-2 + 2.7419x R^2 = 0.961
1/% Inhibition
1/nL

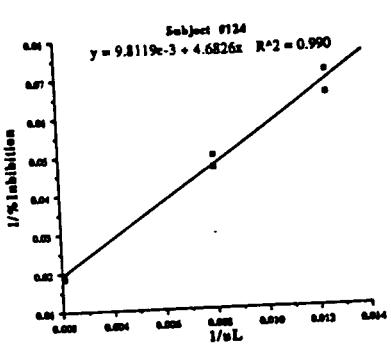

Subject #119
y = 1.0104e-2 + 2.5487x R^2 = 0.990
1/% Inhibition
1/nL

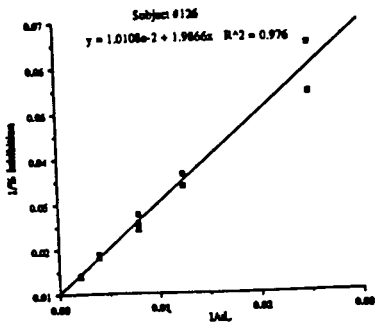

Subject #120
y = 1.0040e-2 + 2.8063x R^2 = 0.980
1/% Inhibition
1/nL

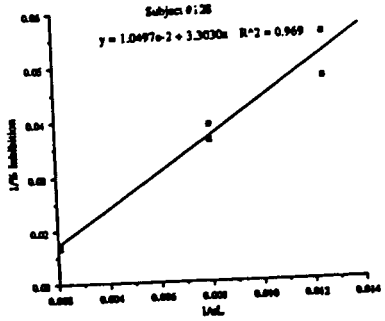

Subject #121
y = 1.0400e-2 + 2.9997x R^2 = 0.943
1/% Inhibition
1/nL

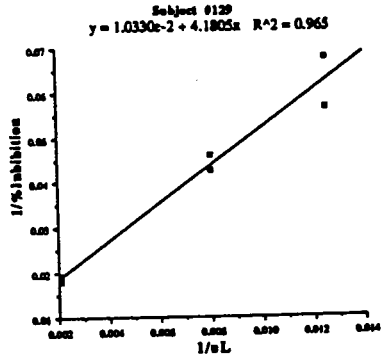

Subject #122
y = 1.0400e-2 + 2.5445x R^2 = 0.997
1/% Inhibition
1/nL

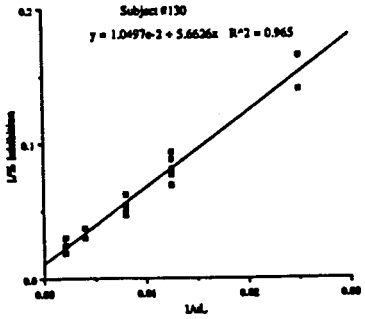

Subject #123
y = 1.0323e-2 + 4.3000x R^2 = 0.988
1/% Inhibition
1/nL

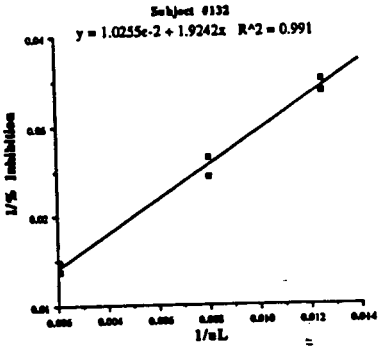

Subject #134
y = 9.8119e-3 + 4.6826x R^2 = 0.990
1/% Inhibition
1/uL

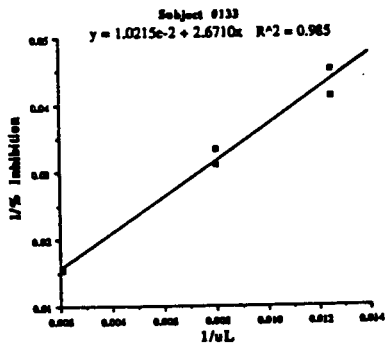

Subject #126
y = 1.0108e-2 + 1.9866x R^2 = 0.976
1/% Inhibition
1/uL

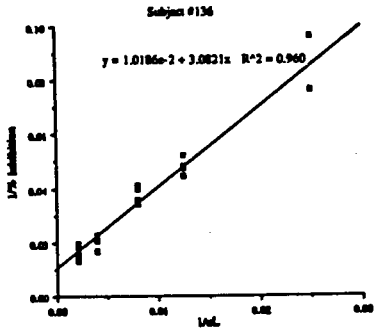

Subject #128
y = 1.0497e-2 + 3.3030x R^2 = 0.969
1/% Inhibition
1/uL

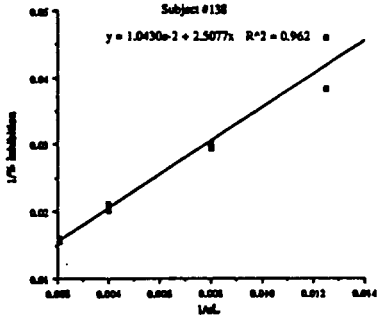

Subject #129
y = 1.0330e-2 + 4.1805x R^2 = 0.965
1/% Inhibition
1/uL

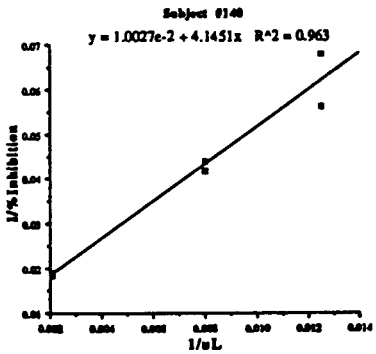

Subject #130
y = 1.0497e-2 + 5.6626x R^2 = 0.965
1/% Inhibition
1/uL

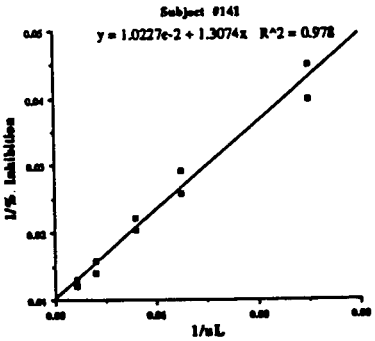

Subject #132
y = 1.0255e-2 + 1.9242x R^2 = 0.991
1/% Inhibition
1/uL


Subject #133
y = 1.0215e-2 + 2.6710x R^2 = 0.985
1/% Inhibition
1/uL


Subject #136
y = 1.0186e-2 + 3.0821x R^2 = 0.960
1/% Inhibition
1/uL


Subject #138
y = 1.0430e-2 + 2.5077x R^2 = 0.962
1/% Inhibition
1/uL


Subject #140
y = 1.0027e-2 + 4.1451x R^2 = 0.963
1/% Inhibition
1/uL


Subject #141
y = 1.0227e-2 + 1.3074x R^2 = 0.978
1/% Inhibition
1/uL

Appendix M

Summary of raw data and SAS corrected values for slope and y-intercept of 1/%Inhibition vs 1/uL SOD plots

Summary of raw data and sas corrected values for slope and y-intercept of 1/%Inhibition vs 1/uL SOD plots

Subject #	y-intercept	y-int SD	y-int T value	y-int P value	slope	slope SD	T value slope	P value slope	corrected slope	corrected slope S.D.	corrected slope T value	corrected slope P value	Dilution factor
4	0.009	0.0028	3.19	0.00100	3.41	0.150	22.80	0.00010	3.38	0.094	35.80	0.00010	1.5
5	0.012	0.0017	6.90	0.00050	1.17	0.065	18.00	0.00010	1.21	0.046	26.30	0.00010	1.5
6	0.012	0.0017	7.21	0.00010	1.40	0.072	19.50	0.00010	1.47	0.051	29.10	0.00010	1.5
7	0.010	0.0028	3.67	0.00630	1.62	0.210	7.68	0.00010	1.62	0.123	13.20	0.00010	1.0
8	0.011	0.0037	2.89	0.01350	2.37	0.154	15.40	0.00010	2.39	0.098	24.50	0.00010	1.5
9	0.011	0.0037	2.95	0.01220	2.31	0.015	15.06	0.00010	2.34	0.097	24.00	0.00010	1.5
11	0.009	0.0039	2.24	0.04450	1.68	0.164	10.25	0.00010	1.64	0.104	15.75	0.00010	1.5
12	0.011	0.0027	4.07	0.00150	1.75	0.114	15.41	0.00010	1.78	0.072	2463.00	0.00010	1.5
14	0.158	0.0992	1.59	0.21000	1.47	3.754	0.39	0.72100	5.58	2.907	1.92	0.12700	1.0
15	0.011	0.0078	1.46	0.17070	2.79	0.329	8.51	0.00010	2.84	0.208	13.62	0.00010	1.0
16	0.011	0.0046	2.47	0.02930	3.30	0.193	17.08	0.00010	3.34	0.123	27.23	0.00010	1.0
17	0.010	0.0027	3.60	0.00570	1.72	0.139	12.43	0.00010	1.71	0.093	18.52	0.00010	1.0
19	0.011	0.0014	7.50	0.00010	1.60	0.099	16.13	0.00010	1.64	0.059	27.65	0.00010	1.0
22	0.011	0.0039	3.14	0.01640	5.21	0.467	11.16	0.00010	5.30	0.241	22.02	0.00010	1.5
23	0.011	0.0034	3.24	0.01020	5.46	0.239	22.89	0.00010	5.52	0.141	39.04	0.00010	1.5
26	0.010	0.0048	2.18	0.06080	3.63	0.444	8.18	0.00010	3.66	0.253	14.48	0.00010	1.5
27	0.020	0.0038	5.10	0.00010	0.31	0.166	1.87	0.08200	0.63	0.121	5.20	0.00010	1.5
28	0.010	0.0027	3.58	0.00500	3.08	0.215	14.31	0.00010	3.06	0.125	24.41	0.00010	1.5
29	0.010	0.0030	3.33	0.00880	3.54	0.284	12.48	0.00100	3.55	0.167	21.30	0.00010	1.5
30	0.011	0.0024	4.56	0.00100	2.43	0.199	12.21	0.00010	2.49	0.123	20.20	0.00010	1.5
31	0.010	0.0042	2.36	0.03750	4.69	0.344	13.60	0.00010	4.69	0.203	23.10	0.00010	1.5
35	0.012	0.0007	18.80	0.00010	1.08	0.058	18.50	0.00010	1.23	0.063	19.50	0.00010	1.0
37	0.010	0.0015	6.30	0.00040	3.98	0.151	26.30	0.00010	3.95	0.101	38.90	0.00010	1.0
38	0.011	0.0032	3.55	0.00530	2.70	0.225	12.00	0.00010	2.78	0.131	21.30	0.00010	1.0
39	0.012	0.0013	9.44	0.00010	2.52	0.134	18.70	0.00010	2.67	0.106	25.10	0.00010	1.0
41	0.011	0.0047	2.35	0.04670	3.12	0.357	8.74	0.00010	3.18	0.209	15.18	0.00010	1.0
42	0.011	0.0119	0.88	0.40100	3.95	0.814	4.85	0.00090	3.97	0.467	8.51	0.00010	1.0
43	0.011	0.0061	1.83	0.10400	3.77	0.463	8.15	0.00010	3.84	0.271	14.15	0.00010	1.0
44	0.010	0.0025	4.00	0.00400	2.39	0.186	12.84	0.00010	2.38	0.109	21.80	0.00010	1.0
45	0.001	0.0023	5.10	0.00030	2.23	0.185	12.10	0.00010	2.34	0.109	21.40	0.00010	1.0
46	0.012	0.0031	3.77	0.00440	2.30	0.246	9.32	0.00010	2.40	0.154	15.59	0.00010	1.0
47	0.011	0.0027	4.10	0.00280	2.78	0.211	13.15	0.00010	2.83	0.130	21.70	0.00010	1.0
48	0.009	0.0013	7.14	0.00010	2.27	0.101	22.30	0.00010	2.21	0.063	35.40	0.00010	1.0
56	0.011	0.0020	5.59	0.00500	1.42	0.150	9.47	0.00010	1.49	0.089	16.59	0.00010	1.5
58	0.009	0.0014	6.70	0.00010	1.22	0.100	12.20	0.10000	1.18	0.060	12.90	0.00010	1.5
59	0.011	0.0058	1.84	0.09800	1.78	0.465	3.82	0.00410	1.82	0.029	6.40	0.00010	1.2
60	0.012	0.0024	4.84	0.00090	2.91	0.190	15.30	0.00010	3.00	0.120	25.10	0.00100	1.5
61	0.011	0.0022	4.80	0.00190	3.82	0.020	19.50	0.00010	3.86	0.114	33.70	0.00010	1.5
62	0.011	0.0036	3.10	0.01200	2.82	0.293	9.60	0.00010	2.89	0.175	16.50	0.00010	1.5
63	0.012	0.0013	8.90	0.00010	2.33	0.111	21.00	0.00010	2.46	0.071	34.70	0.00010	1.5
64	0.010	0.0030	3.25	0.01000	3.54	0.204	17.40	0.00010	3.52	0.117	30.10	0.00010	1.5
65	0.010	0.0042	2.31	0.05000	4.50	0.318	14.10	0.00010	4.48	0.186	24.00	0.00010	1.5
66	0.010	0.0035	2.78	0.02130	2.80	0.274	10.20	0.00010	2.77	0.165	16.80	0.00010	1.5
67	0.011	0.0012	9.27	0.00010	1.71	0.091	18.90	0.00010	1.75	0.054	32.70	0.00010	1.5
68	0.011	0.0017	6.59	0.00010	1.66	0.130	12.80	0.00010	1.72	0.077	22.40	0.00010	1.5
71	0.010	0.0023	4.50	0.00210	2.87	0.174	16.50	0.00010	2.88	0.102	28.30	0.00010	1.5
73	0.010	0.0022	4.80	0.00130	2.82	0.165	17.10	0.00010	2.84	0.097	29.40	0.00010	1.5
74	0.011	0.0014	8.10	0.00010	1.25	0.105	11.80	0.00010	1.32	0.064	20.50	0.00010	1.5

Subject #	y-intercept	y-int SD	y-int T value	y-int P value	slope	slope SD	T value slope	P value slope	corrected slope	corrected slope S.D.	corrected slope T value	corrected slope P value	Dilution factor
76	0.012	0.0043	2.70	0.02700	4.14	0.325	12.70	0.00010	4.23	0.192	22.00	0.00010	1.0
77	0.011	0.0037	2.88	0.01800	3.96	0.253	15.70	0.00010	4.00	0.145	27.50	0.00010	1.5
78	0.010	0.0030	3.52	0.00650	1.53	0.232	6.50	0.00010	1.55	0.135	11.50	0.00010	1.5
85	0.010	0.0011	8.90	0.00010	2.00	0.086	23.40	0.00010	2.01	0.050	40.10	0.00010	1.5
86	0.010	0.0016	6.40	0.00020	2.75	0.123	22.30	0.00010	2.78	0.072	38.30	0.00010	1.5
87	0.011	0.0015	6.80	0.00050	4.15	0.200	20.80	0.00010	4.21	0.096	43.70	0.00010	1.5
88	0.010	0.0016	6.20	0.00010	1.88	0.115	16.30	0.00010	1.88	0.067	27.00	0.00010	1.5
89	0.010	0.0015	6.90	0.00020	1.90	0.096	19.80	0.00010	1.90	0.058	32.60	0.00010	1.5
90	0.010	0.0022	4.60	0.00100	3.62	0.179	20.20	0.00010	3.63	0.106	34.10	0.00010	1.5
91	0.010	0.0012	8.20	0.00010	2.06	0.094	22.10	0.00010	2.07	0.055	37.80	0.00010	1.5
92	0.010	0.0017	6.30	0.00010	4.66	0.126	36.90	0.00010	4.68	0.072	65.40	0.00010	1.5
94	0.011	0.0014	7.80	0.00140	3.90	0.155	25.00	0.00010	3.96	0.070	56.40	0.00010	1.5
95	0.010	0.0001	9.90	0.00060	2.49	0.118	21.00	0.00010	2.51	0.052	47.70	0.00010	1.5
96	0.010	0.0017	6.20	0.00340	3.12	0.193	16.20	0.00010	3.16	0.085	37.00	0.00010	1.5
97	0.011	0.0007	13.80	0.00020	2.89	0.089	32.60	0.00010	2.95	0.042	70.20	0.00010	1.5
98	0.010	0.0010	10.10	0.00010	2.06	0.077	26.80	0.00010	2.03	0.045	45.30	0.00010	1.5
99	0.010	0.0010	10.60	0.00040	2.46	0.113	21.80	0.00010	2.50	0.051	49.30	0.00010	1.5
100	0.010	0.0007	14.80	0.00010	2.73	0.082	33.60	0.00010	2.78	0.038	74.10	0.00010	1.5
101	0.010	0.0029	3.53	0.02430	4.54	0.335	13.50	0.00020	4.56	0.148	30.80	0.00010	1.5
102	0.010	0.0013	8.00	0.00130	3.82	0.235	16.20	0.00010	3.82	0.098	39.00	0.00010	1.5
103	0.010	0.0007	14.30	0.00010	1.44	0.050	28.80	0.00010	1.45	0.030	47.70	0.00010	1.5
104	0.010	0.0013	7.80	0.00010	1.74	0.109	17.40	0.00010	1.75	0.058	30.00	0.00010	1.5
106	0.010	0.0008	13.20	0.00010	1.77	0.060	29.60	0.00010	1.78	0.038	46.80	0.00010	1.5
107	0.012	0.0023	5.00	0.00050	2.77	0.183	15.20	0.00010	2.87	0.109	26.40	0.00010	1.5
109	0.010	0.0019	5.50	0.00150	2.46	0.244	10.10	0.00010	2.51	0.117	21.50	0.00010	1.5
111	0.011	0.0007	17.00	0.00010	2.64	0.074	35.80	0.00010	2.76	0.045	61.70	0.00010	1.5
112	0.010	0.0016	6.20	0.00030	5.85	0.123	45.00	0.00010	5.55	0.072	77.00	0.00010	1.5
113	0.010	0.0015	7.10	0.00010	2.83	0.115	24.70	0.00010	2.86	0.067	42.70	0.00010	1.5
114	0.010	0.0027	3.60	0.00600	5.53	0.213	26.00	0.00010	5.51	0.123	44.60	0.00010	1.5
116	0.010	0.0029	3.60	0.00450	3.38	0.202	16.70	0.00010	3.40	0.116	29.40	0.00010	1.5
118	0.010	0.0026	4.10	0.00340	2.74	0.194	14.10	0.00010	2.77	0.114	24.30	0.00010	1.5
119	0.010	0.0012	8.40	0.00010	2.55	0.091	27.90	0.00010	2.56	0.054	47.80	0.00010	1.5
120	0.010	0.0019	5.30	0.00070	2.81	0.143	19.70	0.00010	2.81	0.084	33.60	0.00010	1.5
121	0.010	0.0031	3.40	0.00850	3.00	0.246	12.20	0.00010	3.02	0.148	20.40	0.00010	1.5
122	0.013	0.0011	11.90	0.00030	2.29	0.125	18.40	0.00010	2.58	0.091	28.30	0.00010	1.5
123	0.010	0.0017	6.10	0.00180	4.30	0.212	20.30	0.00010	4.33	0.106	41.00	0.00010	1.5
124	0.010	0.0021	4.70	0.00910	4.68	0.240	19.50	0.00010	4.66	0.105	44.10	0.00010	1.5
126	0.010	0.0013	7.60	0.00010	1.99	0.104	19.10	0.00010	1.99	0.060	33.00	0.00010	1.5
128	0.011	0.0026	4.10	0.01490	3.30	0.296	11.20	0.00040	3.35	0.131	25.60	0.00010	1.5
129	0.010	0.0035	3.00	0.04020	4.18	0.399	10.50	0.00050	4.21	0.176	23.90	0.00010	1.5
130	0.011	0.0033	3.20	0.00590	5.66	0.279	20.30	0.00010	5.70	0.159	35.70	0.00010	1.2
132	0.010	0.0008	13.10	0.00020	1.92	0.091	21.20	0.00010	1.95	0.041	48.10	0.00010	1.5
133	0.010	0.0015	7.10	0.00210	2.67	0.167	16.00	0.00010	2.69	0.074	36.40	0.00010	1.5
136	0.010	0.0016	6.40	0.00010	3.08	0.148	20.80	0.00010	3.10	0.092	33.70	0.00010	1.5
138	0.010	0.0016	6.70	0.00060	2.51	0.203	12.40	0.00010	2.56	0.097	26.30	0.00010	1.5
140	0.010	0.0085	2.90	0.04640	4.15	0.407	10.20	0.00050	4.15	0.179	23.10	0.00010	1.5
141	0.010	0.0009	11.20	0.00010	1.31	0.070	18.80	0.00010	1.32	0.041	32.30	0.00010	1.5

Appendix N

Summary of experimental parameters and calculated values for all subjects

Subject	Age (years)	Gender	P.A. (kcal/wk)	(kcat Et/Km)'	Lowry [Protein] (ug/mL)	Bradford [Protein] (ug/mL)	[Hb] (g/dL)	Arrhenius slope	units SOD/mL	Units SOD/g Hb
1	24	F					13.91			
2	24	F			4.25	2.18	13.01			
3	25	M	3181		7.71	6.43	12.79			
4	34	M	280	0.465	4.40	3.30	15.65		9303	59466
5	34	M	3036	1.285	12.46		15.62	-969	25703	164602
6	23	M	1176	1.075	12.34		14.98	-652	21495	143513
7	42	M	3712	0.629	5.35	4.06	14.60	-19117	12571	86090
8	46	M	0	0.632	5.89	5.45	17.76	606	12637	71154
9	36	M	3368	0.648	5.16		13.81	3823	12966	93907
10	35	M	236							
11	36	F	3136	0.891	6.00		12.94	518	17822	137756
12	36	F	1750	0.856	7.18	6.86	13.30	-627	17120	126749
13	50	M	1568	0.142			19.90	-1111	2846	14304
14	86	M	1442	0.146			13.95	-2151	2915	20899
15	42	M	133	0.358	2.96		16.36	-118	7158	38969
16	67	M	1232	0.303	3.03		17.28		6059	35063
17	10	M	6566	0.581	7.04		15.26	675	11614	76093
18	42	M	840	0.619	7.76		17.93	638	12382	69046
19	16	M	3964	0.623			16.80		12466	74206
20	53	M	2716		11.83	10.15	10.13			
21	43	M	942	0.288	3.99				5758	
22	79	M	745	0.288	4.17	4.42	13.26	2006	5754	43392
23	64	M	726	0.275	4.09	3.13	16.34	1191	5494	33618
24	17	M	1800							
25	33	M	1564							
26	84	F	1500	0.413	3.33	2.06	12.52		8267	66041
27	40	F	2348	0.381	4.06	4.07	12.55	-7	7626	60776
28	38	M	0	0.488	5.50	4.54	16.88	20	9750	51649
29	42	M	6806	0.423	5.25	5.46	18.13	-167	8467	46708
30	19	M	3490	0.617	7.79	7.57	18.17	1076	12346	67936
31	65	M	2517	0.320	4.53	4.44	18.29	348	6397	34966
32	52	F	1736		9.41	8.10	15.44	-649		
33	36	M	176		10.62	9.86	18.31	-4335		
34	53	F	2520	0.131	2.68	2.00	14.94		2618	17522
35	21	F	24584	0.923	7.99	6.65	12.35	2886	18467	149593
37	67	F	140	0.251	3.89	3.58	15.11		5027	33264
38	66	F	1484	0.370	5.38	4.53	16.40	367	7402	45147
39	59	M	1092	0.397	5.17	4.51	14.97	791	7948	53090
40	61	M	420							
41	61	M	208	0.321	5.25	4.39	21.90	845	8418	29307
42	54	M	7280	0.253	3.80	3.66	15.08		5062	33581
43	58	F	700	0.265	3.24	3.23	14.21		5302	37303
44	57	M	1736	0.416	3.42	3.19	17.85	416	8357	46818
45	43	M	2030	0.448	3.44	3.16	16.15	1427	8960	49365
46	46	M	3080	0.436	4.22	3.38	24.83	1848	6714	35103
47	57	M	2240	0.360	2.61	2.89	16.02	-227	7205	39976
48	61	M	2184	0.442	3.89	3.46	15.62	135	8830	55824
49	38	M	2296							
50	58	F	840							
51	39	M	1317							
52	34	M	308							
53	15	M	12440							
54	11	M	6893							
55	17	M	4920							

Subject	Age (years)	Gender	P.A. (kcal/wk)	(kcal Et/Km)'	Lowry [Protein] (ug/mL)	Bradford [Protein] (ug/mL)	[Hb] (g/dL)	Arrhenius slope	units SOD/mL	Units SOD/g Hb
56	26	M	8850	1.053	11.17	9.21	13.15	280	21067	160238
57	25	F	6222				14.28			
58	27	M	2600	1.230	10.83	10.11	14.44	-372	24598	170377
59	23	F	3520	0.689	7.75	7.08	13.09		13787	105347
60	29	F	5016	0.516	5.52	4.84	15.02	-458	10325	68764
61	80	F	12510	0.393	4.22	3.55	16.27	-969	7857	48297
62	69	M	3980	0.531	3.83	3.50	14.94	-802	10624	71108
63	73	F	4568	0.643	3.84	5.16	14.72	-533	12858	87335
64	71	F	3828	0.424	3.27	1.84	13.26	335	8478	63938
65	83	F	3556	0.333	3.65	3.54	12.59	-2299	6663	52947
66	84	M	1386	0.536	5.74	5.31	17.75	-347	10727	60453
67	17	M	17712	0.679	9.93	6.16	15.41	592	17576	114036
68	21	M	1260	0.905	9.72	7.52	15.85	-451	18097	114197
69	13	F	4016	1.614			15.17	-889	32275	212828
70	51	M	0	1.105	11.62	9.57	13.62	477	22091	159821
71	16	F	644	0.523	6.19	6.57	12.53	-0	10450	83434
73	40	F	140	0.532	6.02	5.73	14.42	-565	10645	73850
74	19	M	8780	1.203	11.25	10.33	16.76	-1169	24082	143544
75	48	F	0		8.07		16.19	638	12382	69046
76	84	M	1284	0.241	2.39	2.08	16.55	-2253	4829	29171
77	61	M	2600	0.379	3.29	2.90	12.75	-669	7576	59419
78	29	M	2656	0.982	9.05	8.33	20.10	-680	19646	97743
79	71	M	3602	1.144	10.76	9.86	17.27	-67	22865	132552
85	20	F	0	0.749	10.27	10.47	13.32	-479	14983	112483
66	47	M	8960	0.545	8.10	6.40	13.70	-534	10904	79620
67	37	F	987	0.362	4.16	4.27	13.98	-604	7237	51765
88	16	M	7859	0.798	6.83	6.21	13.35	98	15965	119589
89	7	F	3560	0.789	6.53	5.36	13.21	128	15778	119461
90	14	F	2262	0.415	3.84	3.21	14.36	-1893	8296	57765
91	17	F	2927	0.727	5.72	4.72	14.36	-770	14540	101236
92	47	F	224	0.322	5.13	4.18	13.53	-1299	6441	47605
94	55	M	952	0.384	5.04	4.22	13.78	348	7685	55782
95	40	M	314	0.602	7.72	6.36	16.67	-649	12047	72291
96	36	M	263	0.480	6.36	5.21	14.84	-4335	9609	64774
97	37	M	406	0.516	6.89	6.21	13.09		10369	79227
98	34	M	392	0.728	7.61	6.80	14.61	-866	14561	99352
99	50	F	0	0.609	5.46	3.94	14.67		12173	82978
100	37	M	1400	0.549	7.39	6.48	19.79	367	10973	55459
101	41	F	672	0.331	4.22	4.49	13.83	791	6611	47800
102	41	F	0	0.393	4.55	4.29	11.01	-1081	7864	71427
103	15	M	3726	1.040	10.70	9.69	14.97	727	20800	138945
104	13	M	5994	0.861	10.54	9.83	14.24	-380	17226	120945
105	50	M	1372	0.965	10.16	8.08	16.69	1362	19295	115626
106	23	M	4508	0.845	8.11	8.72	14.26	-259	16910	118604
107	6	F	0	0.541	7.96	4.55	11.21	-818	10819	96555
109	32	M	3176	0.611	5.88	4.97	17.17	-237	12215	71155
111	82	F	84	0.541	3.56	4.17	14.72	-227	10817	73473
112	87	M	504	0.270	3.87	2.65	18.52	-3104	5407	29201
113	68	M	2606	0.529	5.54	5.94	15.71	-609	10587	67379
114	81	M	728	0.271	3.84	3.83	16.07	-1150	5426	33761
115	80	M	7000	0.879	6.38	7.00	14.57	-549	17585	120736

Subject	Age (years)	Gender	P.A. (kcal/wk)	(kcat Et/Km)'	Lowry [Protein] (ug/mL)	Bradford [Protein] (ug/mL)	[Hb] (g/dL)	Arrhenius slope	units SOD/mL	Units SOD/g Hb
116	68	M	364	0.444	8.81	5.58	14.96	280	8885	59409
118	44	F	644	0.547	4.32	3.42	10.78	2077	10941	101520
119	77	M	112	0.589	7.52	6.32	19.94	-598	11771	59045
120	8	F	0	0.535	6.06	4.56	14.21	-504	10690	75217
121	64	M	6504	0.500	3.26	2.03	17.46	82	10001	57279
122	8	F	0	0.590	4.22	5.62	13.04	-969	11792	90416
123	47	M	2904	0.349	5.00	4.70	14.25	-802	6977	48960
124	39	F	2296	0.320	4.51	3.90	14.74	-533	6407	43472
125	63	F	3500	0.962	11.21	9.57	16.22	-785	19242	118612
126	10	F	3352	0.755	5.63	4.47	17.78	-1535	15101	84922
127	70	M	3176	0.819	7.68	5.31	18.35	-780	16375	89223
128	36	F	308	0.454	5.55	4.31	15.32	592	9063	59306
129	38	M	280	0.359	6.25	4.95	14.81	-451	7176	48447
130	33	F	2974	0.216	2.73	2.21	15.71	-869	4327	27536
131	52	F	358	0.737	7.08	6.52	16.62	477	14742	88700
132	55	F	7000	0.780	8.72	5.74	16.84	-0	15591	92596
133	40	M	364	0.562	5.07	4.13	16.46	-565	11232	68226
134	44	F	314	0.973	10.02	7.81	15.64	-1169	19451	124390
135	47	M	2420	0.681	6.23	4.89	15.68	638	13623	86870
136	33	F	280	0.487	3.18	2.94	14.30	-1274	9734	68091
138	48	F	7000	0.598	5.77	5.44	16.70	218	11963	71657
140	40	M	1092	0.362	5.32	4.20	15.92	-680	7237	45454
141	24	F	13440	1.147	11.35	9.09	12.22	-58	22946	187815
142	78	M	3248	0.851	10.77	9.80	15.29	550	17027	111343
144	47		1052		8.10	6.40	16.8	-534	3141	18922

Appendix O

ANOVA summary of (kcat Et/Km)' for 20 year age groups

Comparison of (kcat Et/Km)' values between age categories

(kcat Et/ Km)'	0-20 years	20-40 years	40-60 years	60-80 years	80-100 years
0-20 years			*	*	*
20-40 years			*	*	*
40-60 years	*	*			
60-80 years	*	*			
80-100 years	*	*			

Note: *denotes a significant difference between compaison groups ($p < 0.05$)

Appendix P

ANOVA summary of Physical Activity for 20 year age groups

Comparison of physical activity levels between age categories

Physical Activity (kcal/wk)	0-20 years	20-40 years	40-60 years	60-80 years	80-100 years
0-20 years			*		*
20-40 years					
40-60 years	*				
60-80 years					
80-100 years	*				

Note: * denotes a significant difference between comparison groups ($p < 0.05$)

Appendix Q

ANOVA summary of Lowry protein for 20 year age groups

Comparison of extract Lowry protein concentration values beween age categories

Lowry protein (ug/mL)	0-20 years	20-40 years	40-60 years	60-80 years	80-100 years
0-20 years			*	*	*
20-40 years				*	*
40-60 years	*				
60-80 years	*	*			
80-100 years	*	*			

Note: *denotes a significant difference betweeen comparison groups ($p < 0.05$)

Appendix R

ANOVA summary of Bradford protein for 20 year age groups

Comparison of extract Bradford protein concentration values between age categories

Bradford protein (ug/ mL)	0-20 years	20-40 years	40-60 years	60-80 years	80-100 years
0-20 years			*	*	*
20-40 years					*
40-60 years	*				
60-80 years	*				*
80-100 years	*	*	*	*	

Note: *denotes a significant difference between comparison groups ($p < 0.05$)

Appendix S

ANOVA summary of Hemoglobin concentration for 20 year age groups

Comparison of hemoglobin concentration values between age categories

Hb (g/dL)	0-20 years	20-40 years	40-60 years	60-80 years	80-100 years
0-20 years				*	
20-40 years				*	
40-60 years					
60-80 years	*	*			
80-100 years					

Note: *denotes a significant differences between comparison groups ($p < 0.05$)

ANOVA summary of (kcat Et/Km)' for Age and Physical Activity groups

Comparison of (kcat Et/Km)' values between subject groups separated for age and physical activity

(kcat Et/Km)'	0-20 Active	0-20 Inactive	20-40 Active	20-40 Inactive	40-60 Active	40-60 Inactive	60-80 Active	60-80 Inactive	80-100 Active	80-100 Inactive
0-20 Active				*	*	*	*	*	*	*
0-20 Inactive										*
20-40 Active				*	*	*	*	*	*	*
20-40 Inactive	*		*					*		*
40-60 Active	*		*							
40-60 Inactive	*		*							
60-80 Active	*		*							
60-80 Inactive	*		*	*						
80-100 Active	*		*							
80-100 Inactive	*	*	*	*						

Note: * denotes a significant difference between comparison groups ($p < 0.05$)

ANOVA summary of Physical Activity for Age and Physical Activity groups

Comparison of physical activity levels between subject groups separated for age and physical activity

Physical Activity (kcal/ wk)	0-20 Active	0-20 Inactive	20-40 Active	20-40 Inactive	40-60 Active	40-60 Inactive	60-80 Active	60-80 Inactive	80-100 Active	80-100 Inactive
0-20 Active		*		*		*		*		*
0-20 Inactive	*		*		*		*		*	
20-40 Active		*		*		*		*		*
20-40 Inactive	*		*		*		*		*	
40-60 Active		*		*		*		*		*
40-60 Inactive	*		*		*		*		*	
60-80 Active		*		*		*		*		*
60-80 Inactive	*		*		*		*		*	
80-100 Active										
80-100 Inactive	*		*		*		*		*	

Note: *denotes a significant difference between comparison groups ($p < 0.05$)

ANOVA summary of Lowry protein for Age and Physical Activity groups

Comparison of extract Lowry protein concentration values between subject groups separated for age and physical activity

Lowry protein (ug/mL)	0-20 Active	0-20 Inactive	20-40 Active	20-40 Inactive	40-60 Active	40-60 Inactive	60-80 Active	60-80 Inactive	80-100 Active	80-100 Inactive
0-20 Active					*			*	*	*
0-20 Inactive									*	*
20-40 Active					*			*	*	*
20-40 Inactive										
40-60 Active	*		*							
40-60 Inactive										
60-80 Active										
60-80 Inactive	*		*							
80-100 Active	*	*	*							
80-100 Inactive	*	*	*							

Note: * denotes a significant difference between comparison groups ($p < 0.05$)

ANOVA summary of Bradford protein for Age and Physical Activity groups

Comparison of extract Bradford protein concentration values beween subject groups separated for age and physical activity

Bradford protein (ug/mL)	0-20 Active	0-20 Inactive	20-40 Active	20-40 Inactive	40-60 Active	40-60 Inactive	60-80 Active	60-80 Inactive	80-100 Active	80-100 Inactive
0-20 Active					*				*	*
0-20 Inactive									*	*
20-40 Active					*				*	*
20-40 Inactive										
40-60 Active	*		*							
40-60 Inactive										
60-80 Active										
60-80 Inactive										
80-100 Active	*	*	*							
80-100 Inactive	*	*	*							

Note: * denotes a significant difference between comparison groups ($p < 0.05$)

ANOVA summary of Hemoglobin concentration for Age and Physical Activity groups

Comparison of hemoglobin concentration values between subject groups separated for age and physical

Hb (g/dL)	0-20 Active	0-20 Inactive	20-40 Active	20-40 Inactive	40-60 Active	40-60 Inactive	60-80 Active	60-80 Inactive	80-100 Active	80-100 Inactive
0-20 Active			*							
0-20 Inactive	*			*	*		*	*		*
20-40 Active				*	*			*		
20-40 Inactive		*							*	
40-60 Active		*	*			*			*	
40-60 Inactive					*			*		
60-80 Active		*								
60-80 Inactive		*	*			*				
80-100 Active					*			*	*	
80-100 Inactive	*									

Note: * denotes a significant difference between comparison groups ($p < 0.05$)

ANOVA summary of Age for Age and Physical Activity groups

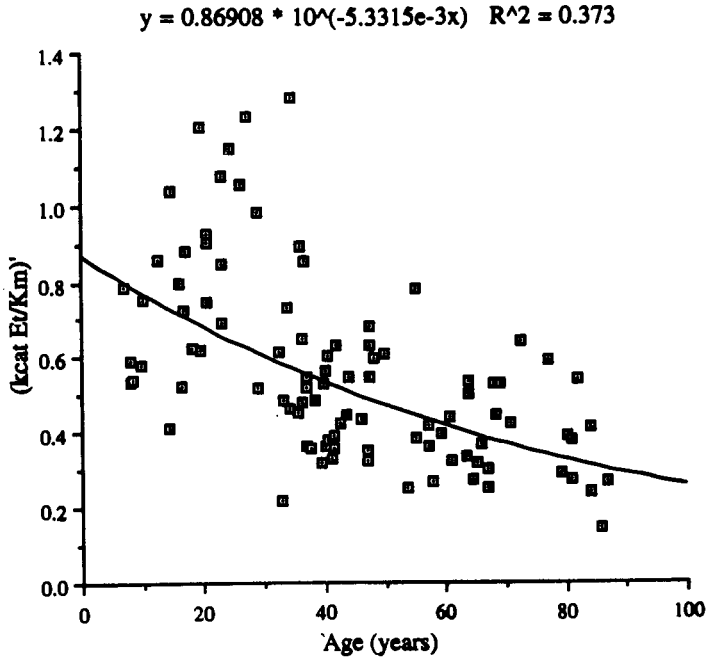
Comparison of age between subject groups separated for age and physical activity

Age (years)	0-20 Active	0-20 Inactive	20-40 Active	20-40 Inactive	40-60 Active	40-60 Inactive	60-80 Active	60-80 Inactive	80-100 Active	80-100 Inactive
0-20 Active			*	*	*	*	*	*	*	*
0-20 Inactive			*	*	*	*	*	*	*	*
20-40 Active	*	*			*	*	*	*	*	*
20-40 Inactive	*	*			*	*	*	*	*	*
40-60 Active	*	*	*	*			*	*	*	*
40-60 Inactive	*	*	*	*			*	*	*	*
60-80 Active	*	*	*	*	*	*			*	*
60-80 Inactive	*	*	*	*	*	*			*	*
80-100 Active	*	*	*	*	*	*	*	*		
80-100 Inactive	*	*	*	*	*	*	*	*		

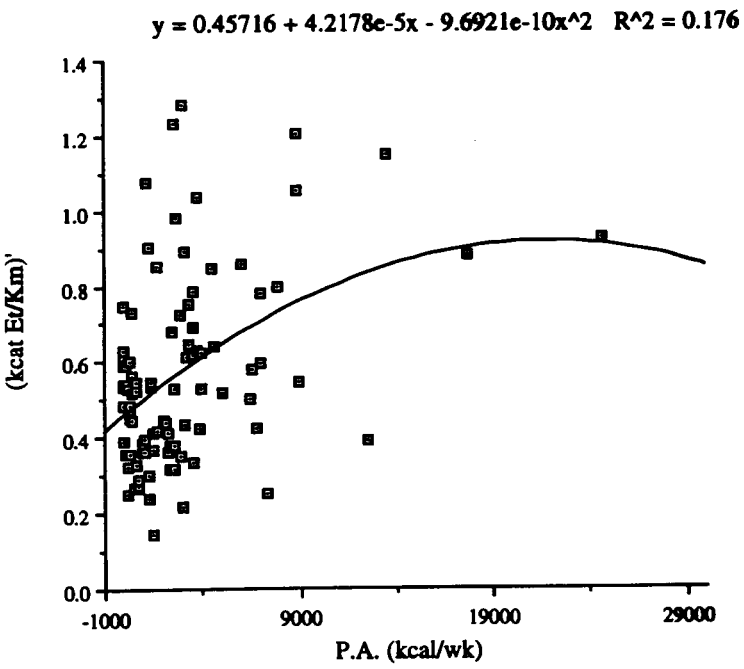
Note: * denotes a significant difference between comparison groups ($p < 0.05$)

Appendix Z

Trend analysis of SOD activity as a function of Age and Physical Activity levels



Relation of SOD activity to age



Relation of SOD activity to physical activity