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LEVELS OF CuZnSOD IN ERYTHROCYTES OF ALZHEIMER'S DISEASE PATIENTS AND NORMATIVE AGING SUBJECTS

by

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THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in the School

of

Kinesiology

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SIMON FRASER UNIVERSITY

January 1995

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ISBN 0-612-06732-7

Canada

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DEGREE: Master of Science

TITLE OF THESIS: LEVELS OF CuZnSOD IN ERYTHROCYTES OF ALZHEIMER'S DISEASE PATIENTS AND NORMATIVE AGING SUBJECTS

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Abstract

The cause of Alzheimer's disease, an age-related progressive degenerative brain disorder, is presently unknown and no cure has yet been found. Pathological features of the disease are quite uniform and indicate that a common mechanism may be responsible for the neurodegeneration. This mechanism may involve increased free radical attack and damage of the brain in Alzheimer's patients. Superoxide dismutase is one of the main antioxidant enzymes in the body.

Twenty-eight individuals diagnosed as having probable Alzheimer's disease (mean age = 74.41 $\pm$ 7.84 years) and thirty-one age-matched neurologically healthy subjects (mean age = 72.07 $\pm$ 8.37 years) took part in this study.

Blood specimens were collected from each subject, a chloroform/ethanol purification (McCord and Fridovich, 1969) was performed to extract the CuZnSOD enzyme, and a CuZnSOD assay was carried out (L'Abbe and Fischer, 1986). To obtain greater accuracy in determining the true value for the activity, multiple points were gathered during the assay for each individual in order to define the line for 1/% inhibition versus 1/volume CuZnSOD extract. Total protein surviving the chloroform/ethanol extraction procedure was measured by both the Lowry method and the Bradford method. Hemoglobin levels in whole blood were also determined. In addition, a temperature study was conducted employing the Arrhenius equation to elucidate a measure of the activation energy of CuZnSOD.

Compared to erythrocytes from the control subjects, the Alzheimer's patients exhibited the following characteristics:

(1) Significantly higher (approximately double) CuZnSOD enzymatic activity (10 798 units SOD activity/mL packed RBCs for the Alzheimer's patients compared to 4 881 units SOD activity/mL packed RBCs in controls; $p<0.05$).

(2) Much lower amount of total protein surviving the harsh chloroform/ethanol extraction as determined by the Lowry method (1.23 $\pm$ 0.47 ug/mL for the Alzheimer's patients compared to 5.34 $\pm$ 2.49

ug/mL for the controls; $p<0.05$). The Bradford method gave similar results.

(3) SDS-gel electrophoresis revealed the presence of very few proteins in the extracts of both subject classes; these were tentatively identified as glutathione reductase, catalase, CuZnSOD, hemoglobin, and calmodulin.

(4) No significant difference was found between hemoglobin values in the two subject groups. Also, CuZnSOD activity showed no correlation with hemoglobin levels.

(5) The relative energy of activation tended to be higher in the Alzheimer's patients extracts compared to the control subjects ($p<0.1$).

The following conclusions may be drawn regarding erythrocytes from Alzheimer's patients compared to control subjects:

(1) Alzheimer's patients have enhanced CuZnSOD synthesis and/or decreased degradation rate of CuZnSOD.

(2) The proteins in the red blood cells of Alzheimer's patients are weaker and more damaged. This increased fragility of proteins may be due to enhanced oxidative stress and protein cross-linking occurring in Alzheimer's patients red blood cells.

(3) The CuZnSOD enzyme is more inhibited (by post-translational modifications) as its catalytic efficiency is diminished.

The enhanced CuZnSOD activity (due to increased synthesis and/or decreased degradation rate of the enzyme), but reduced catalytic efficiency of the enzyme may be a consequence of a compensatory mechanism designed to regulate CuZnSOD activity.

Evidence exists suggesting that too low or too high levels of CuZnSOD may be detrimental to an organism. There appears to be an optimal level for SOD in tissues. Deviations from this level in either direction are deleterious to the individual. If the level of CuZnSOD is too high, as it seems to be in the blood of Alzheimer's patients, it could lead to elevated hydrogen peroxide production. If mechanisms which remove hydrogen peroxide are not similarly increased, hydrogen peroxide may build up. The hydrogen

peroxide molecules could cross the blood-brain barrier. Excess hydrogen peroxide could lead to the generation of hydroxyl radicals through Haber-Weiss or Fenton reactions. Since hydroxyl radicals are very reactive and non-specific in their action, they could damage brain regions. If the assault by reactive oxygen metabolites is severe enough, then neurons may die. Since brain cells are post-mitotic cells, this could be potentially harmful, depending on the degree of neuronal cell loss.

Dedication

To my grandfather, Kashmir Manhas, my father, Karam, my mother, Hardev, my sisters, Sharan and Kiran, and my brothers, Karan and Deepak Manhas, all of whom mean the world to me. May their joys be as deep as the ocean, and their misfortune as light as its foam.

Acknowledgements

I would like to thank my supervisory committee; namely, Dr. N.M.G. Bhakthan, Dr. Lynn Beattie, and Dr. Paul Stankiewicz for their assistance and support in this study.

I am particularly grateful to Dr. Stankiewicz whose stimulating discussions and guidance were extremely valuable.

In addition, I would like to extend my thanks to Dr. Glen Tibbits, whose kindness and advice were greatly appreciated. His benevolence in allowing me to use his lab facilities will not be forgotten.

As well, the generosity of Dr. Allen Tracey, who allowed me to use his spectrophotometer and lab space, cannot go unmentioned.

I am also very grateful to Dr. Andy Hoffer for his assistance in helping to facilitate my project. The concern and interest he took in my work was very encouraging and uplifting.

I am deeply indebted to the staff at the Alzheimer's Clinic at the University of British Columbia for providing blood samples from their patients. Their friendly and cheerful attitude made the interactions pleasant ones.

Also, I will always cherish the love and understanding provided by my family. Their support was instrumental in helping me through sometimes trying times. The advice given by my father was especially useful. I appreciate the help my brothers, Karan and Deepak, and my sister, Kiran, gave me in typing this thesis and the many long hours they put in to assist me.

Finally, a special thanks to all the subjects who participated in this study. Hopefully, their contributions will make a valuable addition to science.

Table of Contents

Approval ii

Abstract iii

Dedication vi

Acknowledgements vii

Table of Contents viii

List of Tables xii

List of Figures xiii

Abbreviations xv

Literature Review 1

- Introduction 1
- Pathological Changes in Alzheimer's Disease Patients 2
- Symptoms of Alzheimer's Disease 3
- What Causes Alzheimer's Disease? 4
- Risk Factors for Alzheimer's Disease 4
- Free Radical Theory of Aging 5
- Aging Phenomena 6
- Free Radicals 8
- Increased Free Radical Formation 11
- Neurotoxins and Free Radicals 11
- Antioxidants 13
    - Superoxide Dismutase 14
- Superoxide Dismutase Activity and Free Radical Damage 15
- Neurons 17

The Blood-Brain Barrier 19

Sensitivity of Neurons to Oxidative Stress 20

The Blood and Brain Connection 21

The Link Between Down's Syndrome and Alzheimer's Disease 23

Chemical Properties of Superoxide Dismutase 26

SOD Family 26

Purification Properties of CuZnSODs 27

Molecular Weight, Subunit Structure, and Metal Content of CuZnSOD 28

Structural Properties of CuZnSOD 29

Stability Properties 30

General Features of the Catalytic Mechanism of CuZnSOD 30

Copper reduction and reoxidation 30

Enzymatic Generation of Superoxide 33

Xanthine Oxidase-Cytochrome C Assay 35

Utilization of Erythrocytes to Determine CuZnSOD Levels 36

Experimental Studies Investigating CuZnSOD Activity in Alzheimer's Disease Patients 37

A Final Word 40

Experimental Aim and Design 41

Hypothesis 41

Purpose 41

Methods 41

Subjects 42

Blood Samples 42

Preparation of CuZnSOD Extract 42

Superoxide Dismutase Assay 43

Protein Determination 46

SDS-Gel Electrophoresis 46

Enzyme Kinetic Analysis 46

Arrhenius Plot 47

Statistical Analysis 48

Results 49

Tables 57

Figures 77

Discussion 111

Evidence for Toxicity Due to Overexpression of SOD 118

Further Support for Oxidative Damage in the Etiology of Alzheimer's Disease 120

Conclusios 126

Future Studies 127

References 128

Appendix A: Aging and Alzheimer's Disease 146

Appendix B: Age-Related Neuronal Changes 147

Appendix C: Why do humans live past their age of optimum health and vigor and into senescence? 149

Appendix D: Evolutionary Considerations 150

Appendix E: The Biological Nature of Aging 152

Appendix F: The Pleiotropic Nature of Oxygen 154

Appendix G: Historical Perspective of the Dual Nature of Oxygen 157

Appendix H: Antioxidants 160

Uric Acid 160

Carotenoids 160

Alpha-tocopherol (Vitamin E) 161

Appendix I: Metals and Alzheimer's Disease 162

Aluminum 162

Calcium 163

Iron 163

Zinc 163

List of Tables

Table 1 Summary of Experimental Parameters 57

Table 2 Summary of raw data and SAS corrected values for slope and y-intercept of 1/% inhibition vs 1/uL SOD plots 58

Table 3 Summary of simple statistics on Alzheimer's patient's experimental parameters 59

Table 4 Summary of simple statistics on control subjects experimental parameters 60

Table 5 Output from SAS Program on Pearson Correlations between experimental parameters 61

Table 6 Summary of correlations present between experimental parameters derived from blood of Alzheimer's patients 65

Table 7 Summary of correlations present between experimental parameters derived from blood of control subjects 66

Table 8 Output from SAS Program on analysis of variance between experimental parameters derived from blood of Alzheimer's patients and control subjects 67

Table 9 Variance between experimental parameters derived from blood of Alzheimer's patients and control subjects 76

List of Figures

Figure 1. Comparison of age (in years) of Alzheimer's Patients and Control Subjects 77

Figure 2. Summary of 1/% Inhibition vs. 1/uL SOD Graphs for Alzheimer's Patients 78

Figure 3. Summary of 1/% Inhibition vs. 1/uL SOD Graphs for Control Subjects 81

Figure 4. Relative Superoxide Dismutase Activities in Erythrocyte Extracts From Alzheimer's Patients and Control Subjects 84

Figure 5. Relation of Relative SOD Activity to Total Activity in Erythrocytes of Alzheimer's Patients 85

Figure 6. Relation of Relative SOD Activity to Total Activity in Erythrocytes of Control Subjects 86

Figure 7. Superoxide Dismutase Activities in Erythrocytes From Alzheimer's Patients and Control Subjects 87

Figure 8. Erythrocyte Relative SOD Activity in Alzheimer's Patients Related to Total Protein Surviving the Chloroform/Ethanol Extraction (Lowry Method) 88

Figure 9. Erythrocyte Relative SOD Activity in Control Subjects Related to Total Protein Surviving the Chloroform/Ethanol Extraction (Lowry Method) 89

Figure 10. Erythrocyte Relative SOD Activity in Alzheimer's Patients Related to Total Protein Surviving the Chloroform/Ethanol Extraction (Bradford Method) 90

Figure 11. Erythrocyte Relative SOD Activity in Control Subjects Related to Total Protein Surviving the Chloroform/Ethanol Extraction (Bradford Method) 91

Figure 12. Relation of Relative SOD Activity (kcat Et/Km)' per Extract Lowry Protein in Alzheimer's Patients and Control Subjects 92

Figure 13. Relation of Relative SOD Activity (kcat Et/Km)' per Extract Bradford Protein in Alzheimer's Patients and Control Subjects 93

Figure 14. Relative Amounts of Lowry Protein Surviving Chloroform/Ethanol Extraction of Erythrocytes From Alzheimer's Patients and Controls 94

Figure 15. Relative Amounts of Bradford Protein Surviving Chloroform/Ethanol Extraction of Erythrocytes From Alzheimer's Patients and Controls ... 95

Figure 16. Relation Between Estimates of Protein Determined by Bradford and Lowry Methods in RBC Extracts ... 96

Figure 17. Erythrocyte SOD Activity Related to Total Protein Surviving Chloroform/Ethanol Extraction in Alzheimer's Patients (Lowry Method) ... 97

Figure 18. Erythrocyte SOD Activity Related to Total Protein Surviving Chloroform/Ethanol Extraction in Control Subjects (Lowry Method) ... 98

Figure 19. Erythrocyte SOD Activity Related to Total Protein Surviving Chloroform/Ethanol Extraction in Alzheimer's Patients (Bradford Method) ... 99

Figure 20. Erythrocyte SOD Activity Related to Total Protein Surviving Chloroform/Ethanol Extraction in Control Subjects (Bradford Method) ... 100

Figure 21. SDS-Gel Electrophoresis of Proteins in Chloroform/Ethanol Extract ... 101

Figure 22. SOD Activity as a Function of Hemoglobin Content in Alzheimer's Patient's Erythrocytes ... 102

Figure 23. SOD Activity as a Function of Hemoglobin Content in Control Subject's Erythrocytes ... 103

Figure 24. Drabkin Hemoglobin Levels in Erythrocytes From Alzheimer's Patients and Control Subjects ... 104

Figure 25. Units of SOD per mL packed RBCs per g Hb as a Function of Extracted Lowry Protein in Alzheimer's Patients ... 105

Figure 26. Units of SOD per mL packed RBCs per g Hb as a Function of Extracted Lowry Protein in Control Subjects ... 106

Figure 27. Units of SOD per mL packed RBCs per g Hb as a Function of Extracted Bradford Protein in Alzheimer's Patients ... 107

Figure 28. Units of SOD per mL packed RBCs per g Hb as a Function of Extracted Bradford Protein in Control Subjects ... 108

Figure 29. Units of SOD per mL Packed RBCs per Gram of Hemoglobin in Erythrocytes from Alzheimer's Patients and Control Subjects ... 109

Figure 30. Relative Arrhenius Slopes for Superoxide Dismutase in Alzheimer's Patients and Control Subjects ... 110

Abbreviations

Å = Angstrom

AD = Alzheimer's disease

Al = aluminum

Arrhenius slope = -Ea

= Temp. slope

ATP = adenosine triphosphate

°C = degrees Celsius

cal = calories

cAMP = cyclic adenosine triphosphate

CAT = catalase

Cu = copper

Cu^{+1} = cuprous

Cu^{+2} = cupric

CuZnSOD = copper, zinc-superoxide dismutase

3-D = three dimensional

4,5-DKT = tryptamine-4,5-dione

DNA = deoxyribonucleic acid

E = enzyme

e^- = electron

Ea = energy of activation

ELISA = enzyme-linked immunosorbent assay

eq'n = equation

Et = total amount of enzyme

Fe = iron

Fe^{+2} = ferrous

Fe^{+3} = ferric

g = gram

GPX = glutathione peroxidase

H^{+} = hydrogen atom

Hb = hemoglobin

H_20 = water

H_2O_2 = hydrogen peroxide

I = inhibition

%I = percent inhibition

%I max = maximal percent inhibition

kcat = enzyme rate constant

kDa = kilodaltons

Km = concentration of substrate which gives half maximal velocity

uL = microlitre

LEP = life span energy potential

ln = natural log

M = moles/Litre

uM = micromolar

Me = metal

Mg = magnesium

mL = millilitres

MLSP = maximum life span potential

mM = millimolar

Mn = manganese

MW = molecular weight

nm = nanometres

O_2 = oxygen

$O_2^{\cdot-}$ = superoxide radical

OH^- = hydroxide ion

$OH^\cdot$ = hydroxyl radical

P = phosphate

R = Universal Gas Constant

RBCs = red blood cells

ROM = reactive oxygen metabolites

rpm = revolutions per minute

[S] = substrate concentration

sec = seconds

SMR = specific metabolic rate

SOD = superoxide dismutase

SOD-1 = copper, zinc-superoxide dismutase gene

Temp. slope = Arrhenius slope

= -Ea

UV = ultraviolet

v = velocity

Vm = maximal velocity

Zn = zinc

LITERATURE REVIEW

Introduction

> "The human brain is the most complex structure in the known universe. The extraordinary properties of this three or so pounds of soft tissue have made it possible for *Homo sapiens* to dominate the earth, change the course of evolution through genetic engineering, walk on the moon, and create art and music of surpassing beauty. Furthermore, the limits of the human mind are unknown." - Richard F. Thompson

One of the crucial arising health problems in societies with a high life expectancy is the increase in age-related neurological diseases. Dementia is a syndrome in adults characterized by a gradual deterioration of intelligence and cognitive ability, often with associated behavioral changes in areas such as self-care. It is present in the elderly whenever the following diagnostic symptoms appear (LaRue et al., 1985): (1) Memory impairment; (2) Impairment in abstract thinking, judgement, or higher cortical functions, or personality change; (3) Loss of intellectual ability severe enough to interfere with social or occupational functioning; (4) Clear state of consciousness (no delirium or intoxication); (5) Documented or presumed evidence of an organic cause.

Alzheimer's disease is the commonest form of dementia in the elderly (Selkoe et al., 1987), accounting for approximately 60% of dementias among people over sixty-five years old (Zarit and Zarit, 1983), and it is the most frequent diagnosis for those entering a hospital with dementia (Wells, 1977). Recent epidemiological data suggest that it affects 5-10% of individuals over 65 years of age and perhaps as many as 40% of those persons over the age of 85 (Evans et al., 1989). Furthermore, it has been estimated that AD is the fourth leading cause of death for

adults in the United States, after heart disease, cancer, and stroke (Katzman, 1986). Given the fact that the population of developed countries is gradually aging as more and more individuals survive to older ages (persons aged 85 years old and over are currently the fastest growing segment of the North American population), it is becoming increasingly apparent that the impact of AD on public health policy will become even greater in the future as the disease becomes more prevalent (Boerrigter, 1992).

Pathological Changes in Alzheimer's Disease Patients

Alzheimer's disease is a complex, progressive, degenerative brain disorder, which shows no social or economic boundaries, and affects men and women almost equally (Rybash et al., 1985). Most victims are over 65 years old; however, the disease can strike in the forties and fifties (Rybash et al., 1985). The unmistakable pattern of slow onset, gradual irreversible losses, and associated changes in brain structure and processes such as neurofibrillary tangles, senile plaques, and neurotransmitter defects (for example, underproduction of acetylcholine) are among its defining characteristics (Cohen, 1988). In addition, amyloid infiltration of cerebrovascular walls is often found in the brains of AD patients (Price, 1986; Kosik, 1991). These lesions are spread throughout the brain, but are most concentrated in the neocortex and hippocampus, and their clinical significance has been highlighted by quantitative studies showing a significant correlation between the numbers of neurofibrillary tangles and senile plaques and the severity of dementia (Riesberg, 1981; Cote, 1981; Scheibel and Wechsler, 1986). It is noteworthy that the presence of plaques and tangles are two prominent features of normal aging. This suggests that there is a quantitative, rather than a qualitative, difference between the brains of the healthy

elderly and those with AD - the brains of AD patients have *more* plaques and tangles than those contained in normal aged brains. Further changes in the brains of individuals with AD include: degeneration in several neuronal systems, such as intrinsic neurons in the neocortex and hippocampus (somatostatin, corticoliperin), cortico-cortical association neurons (glutamate), corticofugul neurons (glutamate), including connections between the entorhinal cortex and hippocampus, as well as ascending projections from the basal forebrain and brain stem to the forebrain (acetylcholine, serotonin, noradrenaline) (Whitehouse et al., 1985; Beal and Martin, 1986; Hyman et al., 1987; Reinikainen et al., 1990).

Symptoms of Alzheimer's Disease

AD is still a diagnosis of exclusion and can only be confirmed at autopsy (with the accuracy of diagnosis normally about 85-90% correct). The symptoms of AD were first described by a German physician, Alois Alzheimer, in 1907 (Alzheimer, 1907). Although the most consistent symptom and finding in patients with AD is memory loss, it is seldom the only feature. A gradual, progressive decline in intellectual function is the hallmark. Symptoms of AD include a gradual memory loss, decline in ability to perform routine tasks, impairment of judgement, disorientation, personality change, difficulty learning, and loss of language skills (Rybash, 1985). There is variation in the rate of change from person to person. The disease eventually renders its victims totally incapable of caring for themselves. Presently, there is no cure for the disease. Death usually ensues after fourteen plus years after the initial onset of symptoms, regardless of the age at which it strikes (Reisberg, 1987).

What Causes Alzheimer's Disease?

The cause of AD is not known and is currently receiving intensive scientific investigation. Both genetic and environmental factors have been proposed to play a role in the pathogenesis (Amaducci et al., 1986; Katzman, 1986). Researchers have identified an abnormal gene on chromosome 21 in families in which AD appears in generation after generation with cyclic regularity (St. George-Hyslop et al., 1989). Furthermore, Rudolph Tanzi and his colleagues (Tanzi et al., 1987) found that the gene responsible for the production of amyloid (the core material of senile plaques) is also located on chromosome 21. However, the majority of cases of AD do not seem to be genetic (or familial) in origin and are unrelated to specific chromosome defects (Ladislave and Crino, 1990).

Nevertheless, the description of several large families in which the occurrence of the disease is consistent with the pattern of transmission of an autosomal dominant gene (familial AD) justifies the assumption that at least in some cases AD is caused by a genetic defect (Goudsmit et al., 1981; Nee et al., 1983). Epidemiologic family studies have generally found a familial incidence of 50% among relatives of AD probands compared to a 10% risk in relatives of controls, indicative of the expected segregational pattern of an autosomal dominant mutation with age-dependent expression (for review, see St. George-Hyslop et al., 1989). Nonetheless, the expression of the genetic defect may be modulated by environmental factors.

Risk Factors for Alzheimer's Disease

Identifying risk factors for AD has been complicated by the potential misdiagnosis of patients and because the patient is often incompetent to provide historical information; thereby,

necessitating information to be obtained from individuals other than the patient. Despite these difficulties, a few putative risk factors have consistently emerged. These include the following: family history of dementia, Down's syndrome, maternal age, head injury, and thyroid disease. Family history of dementia, especially in a sibling, is an important risk factor; however, the relationship between head injury and thyroid disease is less clear (Mayeux et al., 1990).

Free Radical Theory of Aging

While several lines of evidence point to a multifactorial etiology of Alzheimer's disease (Kay, 1987), pathological features of AD are quite uniform and indicate that a single mechanism may be responsible for the progressive brain degeneration. This mechanism may involve increased free radical attack and damage of the brain in AD patients (Volicer and Crino, 1990).

The free radical theory of aging, formulated in November of 1954 by Denham Harman, assumes that there's a single basic cause of aging which is modified by genetic and environmental factors (Harman, 1956). This theory postulates that free radical reactions are involved in aging and disease. It suggests that small, but life-long defects in protection against free radicals cause progressive tissue damage. More specifically, the free radical theory suggests that toxic by-products of oxygen metabolism (oxygen free radicals) react with DNA, proteins, and lipids causing defects in information storage, information retrieval, and enzyme and membrane functions (Harman, 1956). Thus, oxygen metabolism is pleiotropic in nature. It is essential for life in some organisms, but it also has long term detrimental effects. Oxygen is utilized by aerobic organisms due to its ability to efficiently yield a high output of energy compared to anaerobic respiration; however, with the passage of time, oxidative metabolism has

potential negative effects on the organism due to the production of toxic by-products. Support for the free radical theory of aging comes from many areas of study which include the origin and evolution of life, life span experiments (e.g., diet restriction studies), studies implicating free radical reactions in the pathogenesis of specific diseases, and the plausible explanations it provides for aging phenomena.

Aging Phenomena

As stated above, the free radical theory of aging is supported by the plausible explanations it provides for many aging phenomena. These include the following: (1) The observation that average life span in mammalian species is inversely proportional to basal metabolic rate (Cutler, 1982). In fact, the product of specific metabolic rate (SMR) of a species with the maximum life span potential (MLSP) appears to be a constant (termed the life span energy potential; LEP). In other words, for many species "aging rate" is proportional to "metabolic rate" (cal/g/day). Also, animals which hibernate, thereby reducing their metabolic rate since their oxygen consumption is significantly lowered during hibernation, have increased longevity. Furthermore, in lower animals, life span can be extended considerably by keeping cold-blooded animals such as reptiles and insects, at lower temperature, thus decreasing metabolic rate and production of free radicals (Cutler, 1982). Decreasing the metabolic rate of houseflies by preventing them from flying also produces a marked increase in life span (Sohal et al., 1981). (2) The clustering of degenerative diseases in the terminal part of the life span. (3) The exponential nature of the mortality curve. (4) The beneficial effects of caloric restriction on life span and degenerative diseases. Diet restriction studies have shown that both the mean and maximum life span can be extended. Such

experiments use alterations which limit dietary consumption of substances which enhance free radical formation and increase consumption of compounds which inhibit free radical reactions. Increasing dietary consumption of easily oxidized amino acids was found to reduce life span and opposite results were obtained when proteins low in oxidizable amino acids were used as protein sources. In fact, dietary changes have demonstrated (a) an increase in life span in mice, rats, and Drosophila fruit flies; (b) an inhibition of some forms of cancer; (c) a reduction in amyloid formation; and (d) an enhancement of immune function. (5) The increase in autoimmune disorders with age. (6) The decline in mitochondrial numbers, and changes in its structure and function with age. (7) The accumulation of oxidative alterations in long-lived molecules (e.g., chromosomal material, collagen, elastin). It has been demonstrated that low levels of mutagens (which act via free radicals), where the DNA damage induced by the agents appears negligible, cause slow drift of cells away from their proper differentiated state or improper gene regulation. Furthermore, active oxygen species induce chromosomal aberrations, and longer-lived species have a slower rate of accumulation of chromosomal aberrations. (8) The breakdown of mucopolysaccharides via oxidative degeneration. (9) The accumulation of metabolically inert material (i.e., lipofuscin) via oxidative polymerization of lipids and proteins (Harman, 1981). Accumulation of an "age-pigment", lipofuscin, which results from lipid peroxidation, is evidence of cumulative free-radical damage. Lipofuscin accumulates in many, but not all, long-lived, post-mitotic cells, including neurons in the central nervous system and cardiac myocytes (Sohal et al., 1986). Lipofuscin accumulation has been reported to be increased in the olivary nucleus of AD patients (Dowson, 1982), but similar to age-matched controls in the nucleus basalis of Meynert (Mann et al., 1988). It may be significant that in the subpeduncular nucleus, neurofibrillary

tangles were found only in cells with either lipofuscin deposits or in cells containing neuromelanin (Ohm et al., 1988). Melanin itself exists in a free radical form and may catalyze free radical formation (Halliwell et al., 1987). Thus, it is possible that free radicals participate in the development of neurofibrillary tangles which are present in small numbers even in the brains of normal elderly individuals (Rafalowska et al., 1988).

Free Radicals

Chemical bonds are composed of two paired electrons, one with its spin pointing up and the other with its spin pointing down, such that the net spin of the pair of electrons is zero. This is energetically favorable; consequently, electrons prefer to form pairs. Free radicals are species formed by homolytic processes whereby a chemical bond is broken in such a way that one electron goes with each partner. Thus, free radicals have an unpaired electron in an outer shell. They are usually quite reactive (especially small free radicals). However, there is quite a large variation in the degree of reactivity and toxicity of free radicals. Free radicals can be produced in vivo by irradiation (ionizing radiation or light) or by bond homolysis (uncatalyzed or catalyzed) and participate in many biological processes, both beneficial and deleterious to the organism (Halliwell and Gutteridge, 1987; Pryor, 1986; Southorn and Powis, 1988).

Free radicals are generated in the synthesis of eicosanoids and other normal and useful products of oxygenase enzymes. In addition, oxygen free radicals are used by macrophages and leukocytes to kill invading microorganisms as part of the body's defense against infection.

However, free radicals can also be hazardous to host cells and when their concentration exceeds the level that can be handled by the defense systems or when they are in the wrong place

at the wrong time. Free radicals can cause tissue destruction by disrupting membrane functions through peroxidation of lipids, inactivation of enzymes by oxidation of sulfhydryl groups, depolymerization of polysaccharides, and disruption of nucleic acids (Southorn and Powis, 1988).

The involvement of free radicals and reactive oxygen metabolites (ROM), namely superoxide and hydroxyl free radicals, and hydrogen peroxide, and their reactivity with various DNA, lipid, and protein cellular components in the pathogenesis of numerous diseases has become a topic of burgeoning scientific and medical interest (Granger, 1988). ROM have been implicated in several neurological diseases, such as Alzheimer's disease, Parkinson's disease, Down's syndrome, and schizophrenia (Cadet, 1988). Also, inflammatory, carcinogenic, ischemic and immunological diseases, both acute and chronic, are among the diseases in which free radicals are believed to have a hand in the pathogenesis. In addition, free radicals are implicated in atherosclerosis, liver cirrhosis, and cataract formation (Southorn and Powis, 1988). Furthermore, the aging process itself has been proposed to involve the deleterious accumulative effects of increased free radical damage (Harman, 1984; Southorn and Powis, 1988). In fact, Larry W. Oberley (1982) has gone as far as to speculate that:

> "The subject of oxygen radicals in disease will be the most important field of research in medical sciences for some time to come, eclipsing some of the present very popular subject areas. This is because any disease must have as its basis some mechanism of damage, and that mechanism will most likely be shown to be the very reactive oxygen radicals. Thus, oxygen radicals have already been implicated in immune diseases, cancer, aging, diabetes, and muscular dystrophy. Oxygen radicals may not be the initial cause of those diseases, but they may cause the ultimate damage. I predict that knowledge of this area will be essential in future medical treatment."

As already mentioned, oxygen-derived free radicals are formed during several physiological and pathological processes (Halliwell and Gutteridge, 1987; Pryor, 1986). The hydroxy radical, which can be formed from superoxide and hydrogen peroxide by the Haber-Weiss reaction or from hydrogen peroxide in the presence of a cofactor (Fe^{+2} or Cu^{+2}) by the Fenton reaction, is the most reactive of these free radical species (Southorn and Powis, 1988). Free ions of transition metals, such as iron, catalyze the formation of oxygen free radicals (Halliwell and Gutteridge, 1987).

Normally, damage by these highly toxic moieties is prevented or reduced by the action of a defense system consisting of the antioxidant enzymes superoxide dismutase, glutathione peroxidase, and catalase. Superoxide dismutase catalyzes the rapid high affinity conversion of superoxide to hydrogen peroxide. Hydrogen peroxide is then converted to water and oxygen by either glutathione peroxidase or catalase. These three enzymes are present in essentially all tissues of the body. In the central nervous system, the concentrations of superoxide dismutase and glutathione peroxidase are much higher than those of catalase (Marklund et al., 1982). Under certain conditions, however, these regulatory mechanisms are not completely effective and pathological production of free radicals results (Halliwell and Gutteridge, 1987).

Several conditions, such as brain injury, blood flow disturbances, and abnormal free radical metabolism, could increase the rate of the Fenton reaction in the brain and could play a role in the pathogenesis of Alzheimer's disease (Volicer and Crino, 1990).

Increased Free Radical Formation

As stated above, brain trauma is a risk factor for Alzheimer's disease. In fact, a group of researchers have documented the development of Alzheimer's disease pathological changes in a young male after the male sustained brain injury (Rudelli et al., 1982). In addition, the neurofibrillary tangles present in dementia pugilistica (which are thought to result from repeated brain trauma) have been reported to be identical to those observed in the brains of patients with Alzheimer's disease (Roberts, 1988). When the brain is injured, free iron is released and, consequently, free radicals are produced via the Fenton reaction.

A greater than normal amount of free radicals are also formed during reperfusion of ischemic tissue; this excessive free radical production is believed to be the root cause of reperfusion injury (Granger, 1988). Furthermore, amyloid microangiopathy, a condition prevalent in many patients suffering from Alzheimer's disease, primarily affects arterioles and capillaries and could lead to blood flow fluctuations in very small regions of the brain. Although these alterations in blood flow might not cause clinical symptoms, the accumulative effects of repeated ischemia and reperfusion could increase the rate of production of free radicals and result in focal areas of neuronal degeneration (Volicer and Crino, 1990).

Neurotoxins and Free Radicals

Free radicals have also been implicated in the action of some neurotoxins. For example, 6-hydroxydopamine, a catecholaminergic neurotoxin, produces free radicals during its autoxidation. However, the toxicity of 6-hydroxydopamine may also be mediated via an oxidized quinone form which cross-links sulfhydryl groups on proteins (Cohen et al., 1978; Graham,

1978). Indoleamines and catecholamines have been demonstrated to behave as free radical scavengers (DeLange and Glazer, 1988). Interactions between neurotransmitters and free radicals may lead to the generation of endogenous neurotoxin(s). For instance, serotonin has been observed to be oxidized into tryptamine-4,5-dione (4,5-DKT) by the Fenton reaction; 4,5-DKT is a potent neurotoxin which exhibits morphological and biochemical selectivity in the animals studied (Volicer and Crino, 1990).

Furthermore, the induction of stress proteins by neurotoxins and free radical oxidants and the identification of ubiquitin, a small heat shock protein, in the neurofibrillary tangles in Alzheimer's disease provides further support that chronic oxidative stress may play an important role in the pathogenesis of Alzheimer's disease (Lowe et al., 1988). Ubiquitin serves an important role in proteolytic systems aimed at ridding cells of abnormal proteins generated under stress. Overwhelming this capacity may lead to the accumulation of ubiquitinilated protein conjugates in the form of cytoskeletal inclusions (Pappolla et al., 1992). Abnormal cytoskeletal accumulations, known as neurofibrillary tangles, are one of the prominent features of Alzheimer's disease. Studies have revealed that free-radicals are powerful inducers of heat-shock protein synthesis. Cytoskeletal structures are among the primary targets of injury caused by heat shock protein inducers or oxygen free radicals. It has been demonstrated (Pappolla et al., 1991) that the superoxide anion is a powerful inducer of heat shock protein synthesis, and that in response to oxidative stress, intracellular levels of antioxidant enzymes increase several fold (Omar et al., 1987). Under conditions of stress, some heat shock proteins bind tightly to cytoskeletal proteins, thereby, leading to the development of some degree of tolerance to subsequent injury (Wiegant et al., 1987).

Besides the senile plaques, neurofibrillary tangles, and granulovacuolar inclusions, tissue from Alzheimer's disease patients also shows condensed chromatin (Lewis et al., 1981), reduced DNA expression (McLachlan and Lewis, 1985), and increased membrane permeability in several cell types (Zubenko, 1986; Elovaara et al., 1987; Zubenko et al., 1987). These abnormalities are consistent with the expected actions of free radicals.

Antioxidants

Many protective mechanisms have evolved to stabilize the proper differentiated state of cells and reduce the toxicity of oxygen utilization. One method is the compartmentalization of some of the most toxic reactions into a single organelle, such as the mitochondria, which acts to segregate these harmful materials from other more sensitive regions of the cell, such as the nucleus. Another defense mechanism which has evolved is the presence of antioxidants which remove reactive oxygen species before they can damage cellular structures.

In addition, longer-lived species would not be predicted to have different types of protective mechanisms (a qualitative difference) which is more effective, but rather more of the same protective mechanisms (a quantitative difference) (Cutler, 1979). Antioxidants seem to have evolved to reduce the aging rate (not eliminate it) to the extent that aging does not seriously affect the organism's performance before it is killed by other causes unrelated to aging. Many studies have been conducted to determine if longer-lived species do indeed have higher concentrations of antioxidants compared to shorter-lived species. Generally, longer-lived species, particularly humans, do have higher levels of antioxidants per amount of reactive oxygen species produced endogenously (Gerster, 1991). Some of the antioxidants exhibiting a positive

correlation between antioxidant levels and MLSP (maximum life span potential) or LEP (life span energy potential) are the following: superoxide dismutases (CuZnSOD and MnSOD), uric acid, carotenoids, alpha tocopherol (vitamin E), and ceruloplasmin (Cutler, 1986).

Superoxide Dismutase

Superoxide dismutase, a biological antioxidant which removes the superoxide free radicals, is one of the most important enzymes involved in defense against the toxic by-products of oxygen metabolism and without this enzyme (or an equivalent form of protective mechanism), an aerobic organism cannot survive. Experimental studies of mammalian species have found an excellent correlation between the ratio of SOD per SMR and MLSP (Cutler, 1982). Thus, a species' LEP is proportional to the level of SOD in its tissues. The extraordinary linearity of the correlation suggests that this ratio may be a constant, implying that the amount of superoxide free radicals produced per amount of oxygen consumed is similar in mammalian species. Therefore, the maximum amount of oxygen a tissue is capable of utilizing over its life span is directly proportional to the amount of protection provided by SOD that tissue has against the toxic by-products of oxygen metabolism (Cutler, 1986).

The SOD enzyme has a similar structure in many different mammalian species; therefore; the higher concentrations of this enzyme found in longer-lived species is most likely due to more of the SOD enzyme and not the result of a better, more efficient enzyme.

Superoxide Dismutase Activity and Free Radical Damage

As mentioned earlier, the biological effects of oxygen free radicals are controlled in vivo by a wide spectrum of antioxidative defense mechanisms which include cartenoids, alpha tocopherol, metabolites such as uric acid, and antioxidant enzymes. Among these enzymes, the superoxide dismutases catalyze the conversion of the superoxide radical to hydrogen peroxide and oxygen (Fridovich, 1975). Superoxide dismutase, together with catalase (which catalyzes the breakdown of hydrogen peroxide) and glutathione peroxidase (which detoxifies hydrogen peroxide and also converts lipid hydroperoxides into non-toxic alcohols), provide the main defense against damage from harmful oxygen metabolites in human cells.

Oxidative damage in cells can be mediated via the iron-catalyzed Haber-Weiss reaction ($O_2^{\cdot -} + H_2O_2 \rightarrow O_2 + OH^{\cdot} + OH^{-}$) (Halliwell and Gutteridge, 1984). The extremely reactive hydroxyl radicals ($OH^{\cdot}$), thus produced then proceed to initiate a chain reaction which ultimately leads to lipid breakdown of cell membranes (Fee and Teitelbaum, 1972; Brooksbank and Balazs, 1984).

Theoretically, any imbalance in the relative levels of superoxide dismutase, glutathione peroxidase, and catalase may have deleterious effects on cell membranes. Increased superoxide dismutase activity, if not accompanied by similar increases of other antioxidant enzymes, may be harmful. An excess of superoxide dismutase relative to the peroxidases may result in the buildup of hydrogen peroxide. It has been reported (Ceballos et al., 1986) that within the substantia nigra, the CuZnSOD gene is preferentially and highly expressed in the neuromelanin-pigmented neurons. This would facilitate removal of superoxide radicals. Alternatively, a high cellular CuZnSOD activity would promote hydrogen peroxide production which could cause

damage to the neurons. Hydrogen peroxide toxicity due to high superoxide dismutase activity has also been observed in bacteria. An increase in superoxide dismutase activity could lead to production of a large amount of hydrogen peroxide which in the presence of Fe(II) would lead to generation of hydroxyl radicals via the Haber-Weiss reaction or the Fenton reaction (Fe^{+2} + H_2O_2 --> Fe^{+3} + $OH^{\cdot}$ + OH^{-}). Since there is no known defense mechanism against hydroxyl radicals, this could produce damage to brain regions. The deleterious effects of this excessive activity could be due to an increasing formation of hydrogen peroxide and hydroxyl radicals, which are capable of leading to peroxidation reactions, which result in impairments of membrane properties. Thus, a relative increase in superoxide dismutase activity over that of hydrogen peroxide-removing enzymes within the same cellular compartment might result in an increase of oxidative challenge rather than in a greater capability of defense against reactive oxygen derivatives. Similarly, a relative deficiency of superoxide dismutase might lead to the buildup of the superoxide radical, which would shift the Haber-Weiss reaction to the right, resulting in enhanced production of the hydroxyl radical.

Inactivation of SOD-1 has been reported to be associated with aging in rats (Reiss and Gershon, 1976; Glass and Gershom, 1981). Furthermore, induction of superoxide dismutase by oxygen stress was demonstrated to be greater in aerobic bacteria by Gregory and Fridovich and in eukaryotes by Crapo and Tierney. The superoxide dismutase induction capacity is diminished in the elderly compared with that in young adults under conditions of oxidative stress. Experimental studies have also shown that superoxide dismutase induction in leukocytes by in vitro paraquat exposure was greater in healthy subjects aged 20-40 years than in subjects aged 65 to 79 years old (Niwa et al., 1988). Control of SOD-1 activity at the pre-translational level

of gene expression has been documented in both rat and mouse (Delabar et al., 1987). Because active SOD-1 contains both copper and zinc, a lowered SOD-1 activity may reflect copper and/or zinc deficiency (L'Abbe and Fisher, 1986; Okahata et al., 1980; Were et al., 1983), or substitution with another metal ion. Aluminum is of particular interest because elevated levels have been found in the brains of both Alzheimer's disease and Down's syndrome patients (Crapper et al., 1976; Dalton and Crapper, 1977). Oral administration of aluminum hydroxide to rats has been reported to significantly decrease SOD-1 activity in brain (Ohtawa et al., 1983). An aluminum-related reduction in superoxide dismutase activity might account, at least in part, for the additional finding that aluminum salts accelerate the iron-stimulated peroxidation of membrane lipids (Gutteridge et al., 1985).

Thus, either an increase or decrease in superoxide dismutase activity might result in the unscheduled oxidation of susceptible molecules, increased membrane permeability, and cell death. Superoxide dismutase itself, contained in liposomes, has been utilized in the treatment of brain trauma in experimental animals, hence the specific tissue levels of superoxide dismutase necessary for the maintenance of an appropriate redox tissue status is evidently a delicate one.

Neurons

The human brain is thought to consist of one hundred billion neurons, about the same number as the stars in our galaxy. Neurons are the building blocks of the brain. They have the same general organization and the same biochemical apparatus as other cells, but also have some unique characteristics of their own. Among the specialized features that they possess, which other cells do not have, are those which pertain to the special function of neurons as transmitters

of nerve impulses, such as their need to maintain ionic gradients, involving a high rate of energy consumption, and those associated with the ability of neurons to manufacture and release a special array of chemical messengers, called neurotransmitters. Thus, neurons have a distinctive cell shape, have an outer membrane capable of generating nerve impulses, and a unique structure (the synapse) for transferring information from one neuron to the next.

The brain is the most active energy consumer compared to all other organs of the body. This is reflected in its large blood supply and oxygen uptake. Although the human brain represents only two percent of the total body weight, its rate of oxygen utilization (50 mL/min) accounts for twenty percent of the total resting oxygen utilization (Vander et al., 1990). This enormous expenditure of energy is believed to be due to the need to maintain the ionic gradients across the neuronal membrane on which the conduction of impulses in brain neurons depends. In addition, the rate of brain metabolism is relatively constant day and night and may even increase somewhat during the dreaming phases of sleep.

Whereas the other body organs are capable of utilizing a variety of energy sources (e.g., sugars, fats, and amino acids), neurons can only use blood glucose. Furthermore, whereas other tissues such as muscle are able to function for short periods of time in the absence of oxygen, the brain is entirely dependent upon oxidative metabolism. If the oxygen supply is cut off for 4-5 minutes or if glucose is cut off for 10-15 minutes, brain damage will occur. Although elaborate homeostatic mechanisms exist to ensure that the blood pressure will remain stable and that there will be constant levels of oxygen and glucose in the blood, the enormous behavioral flexibility made possible by the expanded size and capacity of the mammalian brain has been acquired during evolution at a high metabolic cost.

The Blood-Brain Barrier

Neurons are extremely sensitive cells. Their function can be disrupted by toxic substances that find their way into the bloodstream and also by small molecules that are normally present in the blood, such as amino acids. The brain is isolated from the general circulation by a selective filtration system called the blood-brain barrier which closely controls both the kinds of substances that enter the extracellular fluid of the brain and the rate at which they enter; thus, regulating the chemical composition of the extracellular fluid of the brain and minimizing the ability of many harmful substances to reach the neurons.

The blood-brain barrier exists within the endothelial cells lining the brain capillaries. Adjacent endothelial cells in brain capillaries are completely sealed together by continuous tight junctions so that all substances entering or leaving the brain must pass through two plasma membranes and the cytoplasm of the endothelial cells (Vander et al., 1990). The blood-brain barrier is selective in what it allows to pass through. Substances which dissolve readily in the lipid components of the plasma membrane enter the brain quickly. This is how some drugs act. Although small molecules such as those of oxygen can pass readily through the barrier, most of the larger molecules required by the brain cells, such as those of glucose, must be actively taken up by special transport mechanisms (i.e., membrane carrier proteins). A similar transport system moves materials out of the brain to prevent buildup of chemical compounds which may interfere with brain function. Thus, the effectiveness of the blood-brain barrier is due to the relative impermeability of the blood vessels in the brain and to the presence of tight sheaths of glial cells

(cells which provide structural and metabolic support for the meshwork of neurons) around the blood vessels.

A few select regions of the brain are not shielded by the blood-brain barrier; they include structures that are specifically responsive to blood-borne hormones or whose job it is to monitor the chemical composition of the blood. These regions, where capillaries in certain areas of the brain are devoid of the blood-brain barrier, account for less than 1% of the brain volume (Vander et al., 1990).

Sensitivity of Neurons to Oxidative Stress

The neurons of the adult brain cannot be replaced and must last a lifetime. Thus, neurons, and other terminally differentiated cells, such as myocytes, which do not replicate in mature animals, are uniquely sensitive to oxidative stress and loss of their function is always serious. Thus, effective function of defense mechanisms against active oxygen is essential. These cells need to survive for many years; therefore, they need to be well protected against active oxygen and reactive oxygen metabolites. Nevertheless, environmental and/or inherited malfunctions are capable of overpowering their defense system. Targets susceptible to oxidative damage on nerve cells include DNA, cell membranes, mitochondrial membranes, axonal membranes, sulphydryl proteins, and microtubules.

Although superoxide and hydrogen peroxide are capable of damaging biological structures to some extent on their own, the major damaging agents are hydroxyl radicals. Hydroxyl radicals show non-specificity in the damage they endow since they abstract the first available hydrogen atom encountered. The most significant damage is likely to happen to DNA molecules or

proteins which have a transition metal ion bound to them (Davison et al., 1988). Since hydrogen peroxide is relatively unreactive, it enjoys a significant life span, and thus is able to diffuse randomly throughout the cell it was generated in and may even traverse a couple of cell membranes. The greatest damage occurs when a reduced transition metal encounters a hydrogen peroxide molecule at an important target, thereby, generating a hydroxyl radical in close proximity to the target (Davison et al., 1988). An electron is transferred to hydrogen peroxide in a Fenton reaction, as follows:

$$Me^{+} + H_2O_2^{-} \dashrightarrow Me^{+2} + OH^{\bullet} + OH^{-}$$

The oxidized metal ion can then be re-reduced by a variety of cellular electron donors. Copper, iron, and manganese are among the metal ions which are effective in intracellular Fenton reactions. An important feature of these metal ions is that they are capable of undergoing redox cycling. In the presence of a source of hydrogen peroxide, their contribution to biological damage is greatly amplified by recurring cycles of reduction followed by hydroxyl radical generation through an ensuing Fenton reaction (Davison et al., 1988).

The Blood and Brain Connection

The vulnerability of the brain to the oxidative stress induced by oxygen free radicals appears to be due to the fact that on the one hand, the brain utilizes approximately 20% of the total oxygen demand of the body and on the other hand, it is not particularly enriched in any of the antioxidant enzymes in comparison to other organs (Harman, 1983; Floyd, 1984).

The *in vitro* measurement of the antioxidant activity of blood sera offers a useful index of redox status. The oxygen free radical metabolizing enzymes in the blood may represent the enzymatic activity in the brain. It could also be that the oxygen free radical metabolizing enzymes in blood have no relation to that present in the brain cells. However, an increase in the oxygen free radical metabolizing enzymes in blood would affect the levels of oxygen free radicals in the brain because the brain is receiving the blood with increased oxygen free radical metabolizing capacity. Red blood cells contain only CuZnSOD, in addition to glutathione peroxidase, which is encoded on chromosome 3 (Wijnen et al., 1978; Johannsmann et al., 1981), and catalase, which is encoded on chromosome 11 (Wieacker et al., 1980; Junien et al., 1980).

Hydrogen peroxide and superoxide are diffusible molecules. If they are produced in excess in red blood cells in Alzheimer's disease, they could diffuse from the red blood cell compartment and cause damage to blood vessels, including the endothelial cells of the brain capillaries via the Haber-Weiss reaction, perhaps contributing to the formation of vascular amyloid. Thus, in Alzheimer's disease, aside from inherent imbalances in the oxidative processes in brain, damage to the brain might be accelerated by continual assault with excessive hydrogen peroxide and superoxide from red blood cells. Damage to the blood-brain barrier might trigger other pathological processes such as autoimmunity or the abnormal accumulation of aluminum that has been documented in the brains of Alzheimer's disease patients (Crapper et al., 1976) and in Down's syndrome patients (Dalton and Crapper, 1976).

The Link Between Down's Syndrome and Alzheimer's Disease

The similarity between the pathological changes observed in Alzheimer's disease patients and Down's syndrome patients suggests that genetic factors are contributing to abnormal free radical metabolism in Alzheimer's disease (Mann, 1988). It is well documented that nearly every individual suffering from Down's syndrome begins to develop the biochemical and pathological symptoms of Alzheimer's disease by about the age of forty years (Kolata, 1985; Wurtman, 1985). Also, there is an increased frequency of Down's syndrome in families of patients with Alzheimer's disease. Down's syndrome patients exhibit chromosome 21 trisomy. Since the gene coding for CuZnSOD is located on chromosome 21, it is overexpressed in Down's syndrome patients.

Because superoxide dismutase plays an important role in the scavenging of oxygen radicals in cells and trisomy 21 patients have three copies of the CuZnSOD (SOD-1) gene, it has been proposed that the extra SOD-1 gene in Down's syndrome is responsible for some of the manifestations of Down's syndrome (SOD-1 in humans has been localized to band 21q22.1 on the long arm of chromosome 21). The increase in SOD-1 in Down's syndrome due to chromosome 21 trisomy suggests that disturbances in the metabolism of reactive oxygen metabolites and enhanced lipid peroxidation may contribute to the distinctive pathological features of the disease. In addition to increasing lipid peroxidation in the brains of Down's syndrome patients (Brooksbank and Balazs, 1984), the unusually rapid elimination of superoxide radicals may affect the oxyradical biosynthesis of neuromediators (Michelson et al., 1977; Sinet, 1982; Jezioroska et al., 1988). Increased levels of SOD activity will result in an increased rate of conversion of superoxide radicals into hydrogen peroxide. If the ability of enzymes to remove

hydrogen peroxide is overloaded due to the presence of excessive hydrogen peroxide, then the pathway to hydroxyl radical formation will be favored. Recall that hydrogen peroxide is a substrate for both the Fenton reaction and the Haber-Weiss reaction which both lead to the production of the extremely destructive hydroxyl radicals which are capable of reeking havoc in biological systems due to their high, non-specific reactivity (Sinet, 1979).

In fact, there is considerable evidence suggesting increased damage from oxidative processes in trisomy 21 (Sinet, 1982; Brooksbank and Balazs, 1984). Although direct evidence implicating increased SOD activity in the pathogenesis of Down's syndrome is lacking, convincing evidence exists supporting the notion that elevated SOD activity mimics some of the Down's syndrome features (Volicer and Crino, 1990). A study involving transgenic mice carrying the human CuZnSOD gene found that the mice expressing elevated levels of h-CuZnSOD displayed pathological changes at the tongue neuromuscular junction which are similar to defects observed in the tongue muscle of Down's syndrome patients (Avraham et al., 1988; Yarom et al., 1988). Another similarity between these transgenic mice and Down's syndrome patients was that like the platelets of individuals with Down's syndrome (Coyle, 1986), the blood platelets from the transgenic mice were observed to have a less efficient uptake mechanism for serotonin (Schickler et al., 1989). This defect is due to a reduction in the transport rate of serotonin into dense granules in both the Down's syndrome platelets and platelets from the transgenic mice. Also, a study found that glutathione peroxidase (which detoxifies hydrogen peroxide) activity was elevated in erythrocytes of Down's syndrome patients (Sinet, 1979), thus providing further evidence that Down's syndrome patients are exposed to higher free radical concentrations compared to neurologically healthy individuals. The increase in glutathione

peroxidase activity observed may reflect a compensatory adaptation to increased hydrogen peroxide load. In addition, an experiment which involved the insertion of an extra copy of the CuZnSOD gene into normal fibroblasts resulted in increased lipid peroxidation (Groner, 1986).

Involvement of chromosome 21 in Alzheimer's disease is not clear. A locus for familial Alzheimer's disease has been located on chromosome 21, but there is no convincing evidence for an excess of chromosome 21 DNA in Alzheimer's disease. It has been reported that superoxide dismutase activity is increased in some brain regions of Alzheimer's disease patients (Marklund et al., 1985) and in fibroblasts of Alzheimer's disease patients (Zemlan et al., 1989). However, recent evidence indicates that there are multiple genetic loci for Alzheimer's disease (Bird et al., 1989). Chromosome 21 is not the only chromosome implicated in Alzheimer's disease. Alzheimer's disease appears to be a heterogenous disorder and familial forms are heterogenous. Discordance for Alzheimer's disease in identical twins suggests that additional non-genetic factor(s) are required for the development of the disease (Nee et al., 1987). Brain injury in an individual with genetic abnormalities of free radical metabolism may lead to formation of endogenous neurotoxin(s) which initiate progressive brain degeneration. Initial formation of a small amount of a neurotoxin could lead to further brain damage and release of more free radicals, establishing a self-propagating cycle. In contrast, multiple distinct insults may initiate Alzheimer's disease even in individuals without genetic predisposition.

Chemical Properties of Superoxide Dismutase

The study of oxygen free radicals is built upon the work of Dr. Irwin Fridovich. In 1969 (McCord and Fridovich), the biochemical community was introduced to a new enzyme, conceived in mystery and dedicated to the abolition of superoxide free radicals. Although Fridovich was not the first to purify superoxide dismutase, he was the first to explain its function and significance.

SOD Family

There are three forms of the superoxide dismutase enzyme in mammalian systems. Table 1 indicates the general properties of the mammalian isozymes. These forms segregate into two families, the CuZn and Mn/Fe dismutases, with dramatically different properties. It appears that these two families have evolved from different ancestral proteins and arose in different areas during the evolution of species.

The three isozymes of superoxide dismutase which have so far been identified are the following: the cytoplasmic form, CuZnSOD (SOD-1), which is encoded on chromosome 21 (Tan et al., 1973; Philip et al., 1978; Levanon et al., 1985); the mitochondrial form, MnSOD, which is encoded on chromosome 6 (Creagen et al., 1973); and the extracellular SOD, which is believed to have a separate gene locus (Marklund, 1984).

Prokaryotes, both bacteria and blue-green algae, contain only enzymes of the Mn/Fe family. The CuZn forms are found in all higher eukaryotes, from fungi, ferns, mosses, and land plants, to invertebrates and vertebrates, while protozoans, the most primitive eukaryotes, lack CuZn superoxide dismutases (Asada et al., 1980). The first appearance of the CuZn form has

been pinpointed to within the eukaryote green algae, of the class Chlorophyceae (Henry and Hall, 1977). Since the earth originally contained a reducing environment, it is likely that superoxide dismutase (acting on an oxygen by-product) would have evolved after the accumulation of atmospheric oxygen was sufficient to constitute an oxygen toxicity threat.

Of the four CuZn superoxide dismutases sequenced, 12 of the first 40 residues are invariant over a vast phylogenetic range, and an identity of nearly 50% is found when the entire amino acid sequences are compared (Barra et al., 1980). This indicates that the members of the CuZn superoxide dismutase family have diverged very slowly, because their sequence conservation is so great (Barra et al., 1980). Such conservation is considered characteristic of proteins with a crucial physiological role, and is consistent with the protective function proposed for dismutases.

Purification Properties of CuZnSODs

It is the stability difference which distinguish the two superoxide dismutase families more strikingly than differences in structural parameters (Steinman, 1982). When purifying CuZnSOD, the crude homogenate is treated with a chloroform-ethanol mixture, and the supernate obtained after centrifugation is saturated with dibasic potassium phosphate, effecting a separation of organic and aqueous phases. The CuZn superoxide dismutase partitions into the aqueous phase, from which it is precipitated by addition of acetone. The ability to withstand such harsh treatment classifies CuZn superoxide dismutases as one of the most stable enzyme families and also contributes greatly to the ease with which these enzymes are purified, because many other

proteins are irreversibly denatured or precipitated by this treatment (McCord and Fridovich, 1969; McCord et al., 1977).

The purification of many CuZn-dismutases has been performed with such protocol: chloroform-ethanol treatment of the homogenate, followed by salting out of the organic phase, and acetone precipitation. This protocol, which is an adaptation of the Tsuchihashi method for removing hemoglobin from erythrocyte lysates (Tsuchihashi, 1923), is referred to as the McCord-Fridovich method, after its use in isolation of the first superoxide dismutase (McCord and Fridovich, 1969).

Molecular Weight, Subunit Structure, and Metal Content of CuZnSOD

The CuZn dismutases are a remarkably conserved family with respect to their gross structural properties (Fridovich, 1978; Crapo et al., 1978; Fridovich, 1974). Without exception, the purified enzymes have been shown to be dimers (native molecular weight 31, 000 to 33, 000) containing very nearly 2.0 g-atoms of copper and 2.0 g-atoms of zinc per mole. The two subunits are apparently identical and are associated solely by noncovalent interactions. Subunit molecular weights estimated by physical methods (15, 000 to 17, 000) are in good agreement with the results of complete amino acid sequences now known for the enzymes from bovine (Evans et al., 1974; Steinman et al., 1974; Abernethy et al., 1974) and human (Barra et al., 1980) erythrocytes. Each subunit contains one intrachain disulfide bond and one active site, containing an atom of copper and an atom of zinc. Both the intrasubunit disulfide bonds and the metal-binding sites contribute to the strength of the subunit association.

CuZnSODs show two rather constant and characteristic features in their amino acid compositions. First, the content of tyrosine and tryptophan is very low (Barra et al., 1980; Jabusch et al., 1980). Human erythrocyte CuZnSOD contains only one tryptophan per subunit. Second, the content of glycine is high, from 13 to 17 mol%, corresponding to approximately 20 to 26 residues per subunit chain, or an average of about one glycine per every 6 to 8 amino acid residues (Steinman et al., 1974; Barra et al., 1980; Jabusch et al., 1980). Glycine is distributed rather evenly throughout the chain in the four known sequences.

In the 3-D structure of the bovine CuZnSOD, this high content of glycine appears to be essential for the extensive beta-pleated sheet conformation, with intervening sharp bends in the polypeptide, and it is probably for this reason that this generous endowment with glycine has been conserved throughout the evolutionary history of CuZn-dismutases (Richardson et al., 1975).

Structural Properties of CuZnSOD

The structural properties of CuZnSOD from bovine erythrocyte has been completely worked out. The two subunits of the dimer, known to be chemically identical, appear to have identical, or nearly identical, backbone conformations at 3 Å resolution. The predominant feature of the structure is its large amount of beta-sheet. This sheet contains nearly 50% of the sequence, in 8 strands of antiparallel arrangement. In three dimensions, the strands comprise a cylinder or barrel. At the subunit interface, and in many of the turns between beta-strands, there reside glycine residues which are probably crucial for closeness of approach and sharp bending, respectively. The metal-binding sites for copper and zinc are on the opposite side of the molecule from the subunit interface. The two copper ions on the two separate subunits are

approximately 34 Å apart. The copper and zinc ions within each subunit are only about 6 Å distant, and are liganded by imidazole and carboxyl side-chain functions from residues in the beta-sheet and loop structures of the molecule. The metal(s) in the CuZn superoxide dismutase serve to stabilize and organize secondary structural elements of the holoprotein, many of which already exist in the apoprotein.

Stability Properties

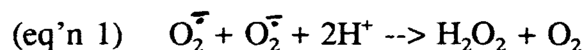
The stability of the enzyme from bovine erythrocytes has been documented more extensively than that of any other CuZnSODs. The activity (second-order rate constant for catalysis) is constant over a range from pH 4.5 to pH 9.5. Also, the bovine enzyme is stable to repeated freeze-thaw cycles, and to prolonged refrigeration. However, while CuZn superoxide dismutases are a remarkably stable enzyme family with respect to their stability to the McCord-Fridovich protocol, it may be anticipated that some species variation from the paradigm of the bovine erythrocyte protein may be encountered. In appreciation of this variability, it is prudent to avoid indiscriminate exposure to extremes of denaturing or dissociating agents, to heat, or pH.

General Features of the Catalytic Mechanism of CuZnSOD

Copper reduction and reoxidation

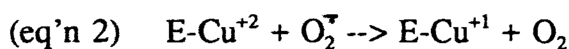
At neutral pH the superoxide radical is predominantly in the anionic form, O_2^-, being the conjugate base of the hydroperoxyl radical ($HO_2^{\cdot}$), whose pKa is 4.8 (Behar et al., 1970). Any

catalyst must circumvent the electrostatic repulsion and consequent slow reaction between the two superoxide anions indicated in the chemical equation (eq'n 1) (Fridovich, 1979).

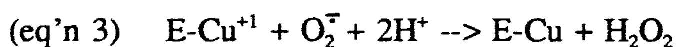


$$\text{(eq'n 1)} \quad O_2^{\bar{\cdot}} + O_2^{\bar{\cdot}} + 2H^+ \dashrightarrow H_2O_2 + O_2$$

The copper of superoxide dismutase acts as an electron carrier between the superoxide radicals, in two successive half-reactions, and the two anions never actually meet. This mechanism is depicted in equations 2 and 3 (Fridovich, 1979).



$$\text{(eq'n 2)} \quad E\text{-}Cu^{+2} + O_2^{\bar{\cdot}} \dashrightarrow E\text{-}Cu^{+1} + O_2$$



$$\text{(eq'n 3)} \quad E\text{-}Cu^{+1} + O_2^{\bar{\cdot}} + 2H^+ \dashrightarrow E\text{-}Cu + H_2O_2$$

E = one superoxide dismutase subunit

The resting enzyme contains copper in the oxidized (Cu^{+2}) state, deduced from x-ray absorption studies (Blumberg et al., 1978). In the first half-reaction, enzyme-bound copper is reduced from the cupric to the cuprous state, and the first superoxide radical is oxidized to dioxygen (eq'n 2) (Steinman, 1982). In the second half-reaction, copper is reoxidized to cupric, while the second superoxide is simultaneously reduced and protonated to form hydrogen peroxide (eq'n 3). Thus, the first half-reaction involves only one electron transfer, whereas the second half reaction requires both electron and proton transfer. The source of the proton is not known for certain, and has been the subject of some investigation (Steinman, 1982).

From monitoring the disappearance of superoxide, by UV absorbance measurements of irradiated enzyme solutions, the second-order rate constant for catalysis has been found to be 2 X 10^9 M^{-1} sec^{-1} (Klug et al., 1972; Rotilio et al., 1972). An identical value has been found for both bovine and human erythrocyte dismutases, and the constancy of this value has been shown over the range of pH 4.8 - 9.5 for the bovine enzyme and pH 5.7 - 10.5 for the human enzyme (Klug et al., 1972; Rotilio et al., 1972; Bannister et al., 1973). Studies indicate that the rate of the enzyme catalyzed reaction is diffusion limited (Rotilio et al., 1972; Fielden et al., 1974). From monitoring at a wavelength near the absorbance maximum of the enzyme-bound copper, it is possible to measure (1) the rate at which superoxide reduces the oxidized (i.e., "resting") enzyme to the cuprous form, and (2) the rate at which superoxide oxidizes the reduced enzyme (formed by treating resting enzyme with hydrogen peroxide) to the cupric state (Fielden et al., 1974). X-ray absorption spectroscopy has established that the valence of Zn^{+2} does not change during catalysis, and that on reduction, the valence of enzyme-bound copper changes from Cu^{+2} to Cu^{+1} (Blumberg et al., 1978). Both superoxide and the cupric form of superoxide dismutase are paramagnetic.

The available evidence, obtained on bovine superoxide dismutase, supports a mechanism in which the superoxide anion displaces a water molecule from the inner coordination sphere of copper, prior to or during electron transfer, in both the reduction and oxidation steps of dismutation (Steinman, 1982).

Copper is essential for activity; all metal replacements of it have rendered the enzyme totally inactive and reversible removal of copper has been associated with reversible loss of activity (McCord and Fridovich, 1969). Zinc, on the other hand, is dispensable. Thus, copper-

containing proteins, in which zinc has been replaced by divalent mercury, cobalt, or cadmium, have activities identical or nearly identical (70% to 90%) to that of the native copper-zinc protein (Beem et al., 1974). It appears that the zinc ions role is to maintain the stability of the enzyme.

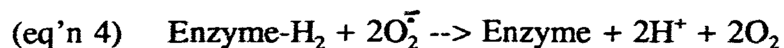
Enzymatic Generation of Superoxide

Of the many enzymes which generate superoxide radicals during enzymatic activity, only a few are of practical use for the study of superoxide. These are the oxidoreductases which generate free superoxide, which can diffuse from the point of origin (active site) into the bulk of the solution for further reaction. One of the proven enzymes to generate free superoxide is xanthine oxidase (xanthine: oxygen oxidoreductase 1.2.3.2) (Fridovich, 1970; Olson, 1974a; Olson, 1974b). Presently, it is the most frequently used enzyme because of its commercial availability in relatively pure form.

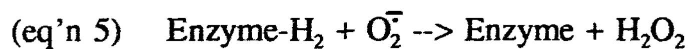
Xanthine oxidase is a very complex enzyme which contains 2 moles of flavin adenine dinucleotide (FAD), 8 moles of iron, 2 moles of molybdenum, and labile sulfide groups (Allen and Bielski, 1982). Its catalytic specificity is low both with respect to substrate oxidation (hypoxanthine, xanthine, pyrimidines, aldehydes, pterins, reduced nicotinamide adenine dinucleotide (NADH), etc.) and electron acceptors (oxygen, ferricyanide, artificial dyes) (Allen and Bielski, 1982). Although this is one of the most extensively studied enzymes, how it transfers electrons from donor to acceptor is not yet well understood.

In the standard laboratory method (Fridovich, 1970) for superoxide radical generation, the preferred substrates are either xanthine or acetaldehyde, which are oxidized to uric acid and acetic acid, respectively. In the enzymatic process, reduced xanthine oxidase loses electrons by two

different pathways, a univalent reaction yielding superoxide (eq'n 4) and a divalent reaction which leads directly to hydrogen peroxide formation (eq'n 5) (Allen and Bielski, 1982).



(eq'n 4) $\text{Enzyme-H}_2 + 2O_2^{\bar{\cdot}} \text{ --> Enzyme} + 2H^+ + 2O_2$



(eq'n 5) $\text{Enzyme-H}_2 + O_2^{\bar{\cdot}} \text{ --> Enzyme} + H_2O_2$

The percentage yield of the superoxide radical in this system has been found to vary from 20% to 100% depending upon pH, oxygen concentration, and the turnover rate of the enzyme. In general, raising the pH and oxygen concentration or lowering the turnover rate favors the reaction illustrated in eq'n 4 (Allen and Bielski, 1982).

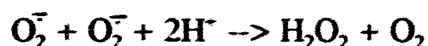
Although the xanthine oxidase system is a very useful source of superoxide radicals for steady-state studies, great caution should be employed in its use. The presence of hydrogen peroxide and inherent small amounts of metallic impurities in such enzyme systems can easily lead to Fenton-type ($Fe^{+2} + H_2O_2 \text{ --> } Fe^{+3} + OH^{\cdot} + OH^-$) reactions and; therefore, to misinterpretation of observed results.

Superoxide dismutases are unique in that their substrate is an unstable free radical. Because the enzyme substrate and the products are unstable, it is difficult to measure the disappearance of substrate or the formation of products as is usual in enzymatic assays. It is not possible to use the normal routine of preparing a stable stock solution of the substrate and then monitoring the acceleration of its conversion to product by the enzyme. Thus, it is necessary to devise strategies to circumvent the instability of the substrate radical for assaying the activity of superoxide dismutase. Routine assays for superoxide dismuatase usually employ an indirect

assay. The most frequently used method for measuring superoxide dismutase activity utilizes the xanthine/xanthine oxidase reaction for superoxide generation and reduction of cytochrome c or nitroblue tetrazolium for superoxide detection.

Xanthine Oxidase-Cytochrome C Assay

Xanthine oxidase reduces molecular oxygen to hydrogen peroxide. The precursor of the $2e^-$ reduced form of molecular oxygen (i.e., hydrogen peroxide) is the $1e^-$ reduced form (i.e., the superoxide radical). Xanthine oxidase can reduce cytochrome C, but only in the presence of superoxide. Molecular oxygen and cytochrome C could normally be thought of as competing electron acceptors, so an oxygen requirement for cytochrome C is very peculiar. Hydrogen peroxide is not responsible for the reduction of cytochrome C by the aerobic xanthine oxidase reaction, rather the $1e^-$ reduced form of oxygen is. Superoxide dismutase is a thermolabile inhibitor of cytochrome c reduction, which can compete with cytochrome C for the flux of superoxide generated by xanthine oxidase:



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

Cytochrome C does not bind to xanthine oxidase. Instead, it is reduced in free solution by superoxide released by xanthine oxidase. Superoxide dismutase operates by intercepting superoxide in free solution, facilitating the dismutation of superoxide. The inhibition of the reduction of cytochrome C during the xanthine oxidase reaction has proved to be a convenient assay for superoxide dismutase. One unit of superoxide dismutase activity has arbitrarily been

defined as that amount which causes 50% inhibition of the xanthine oxidase system under specified conditions.

Utilization of Erythrocytes to Determine CuZnSOD Levels

Erythrocytes have many characteristics which make them advantageous for studying CuZnSOD levels and the degree of oxidative stress in an organism.

Since the main function of red blood cells is to transfer oxygen to tissues, it is exposed to high oxidative stress levels. Erythrocyte membranes are characterized by a high content of polyunsaturated fatty acids. The extent of lipid peroxidation in erythrocytes exhibits an age-related increase (Joswiak and Jasnowska, 1985). Lipid peroxidation of red blood cell membranes initiates numerous irreversible damage. For example, a major by-product of lipid peroxidation, called malondialdehyde, accumulates and causes 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 function and stability of hemoglobin (Kikugawa et al., 1984). The degree of oxidative stress is readily determined by measuring malondialdehyde content (Joswiak and Jasnowska, 1985).

Furthermore, throughout the life span of erythrocytes, no detectable protein turnover occurs. Thus, erythrocytes offer a good model for the study of free radical-induced damage as a function of long-term exposure of proteins to the in vivo environment. The life span of red blood cells in young human adults is 110-120 days (Glass and Gershon, 1981). However, the turnover rate of red blood cells in circulation increases with age; therefore, the life span of erythrocytes shortens as an animal ages.

In addition, red blood cells only contain CuZnSOD; thus, contamination with MnSOD is not a problem. The CuZnSOD in erythrocytes provides protection against the oxidation of hemoglobin, peroxidation of membrane lipids, and superoxide induced hemolysis.

Finally, CuZnSOD is abundant in red blood cells (Winterbourn et al., 1975).

Experimental Studies Investigating CuZnSOD Activity in Alzheimer's Disease Patients

The literature contains conflicting reports as to what changes, if any, are occurring in CuZnSOD activity in Alzheimer's disease patients compared to neurologically healthy aged adults. Not only are the results of different studies sometimes conflicting, but also they are sometimes difficult to interpret. These conflicting results are likely due to differences in the populations studied, age and sex of the subjects, age classifications, methods of sampling, sample size, the way samples are prepared, assay methods, conditions of the assay, and definition or representation of activity (Abe et al., 1989).

Perrin et al. (1990) assayed CuZnSOD in erythrocytes obtained from 25 institutionalized patients with Alzheimer's disease (20 females and 5 males; aged 74-98 years; mean age = 87.9 ± 6.8 years), 25 age-matched institutionalized patients showing no neurological symptoms (22 females and 3 males; aged 74-98 years; mean age = 88.4 ± 7.1 years), and a reference group formed by 41 supposedly healthy men and women (aged 49-63 years; mean age = 54.7 ± 3.6 years). They found that CuZnSOD activity measured in the erythrocytes of the elderly was decreased if compared to the younger reference group, while a significant elevation was observed in erythrocyte CuZnSOD activity in the Alzheimer's disease patients compared to the non-

Alzheimer's disease patients. However, SOD activity was expressed as units per hemoglobin (Hb) content of the hemolysates. Yet it has been demonstrated that hemoglobin levels themselves are not constant, but vary with respect to age. It has been observed that cell volume and cell hemoglobin concentration decrease with both increased cell age and animal age (Abraham et al., 1978). Most researchers tend to express their data in units per gram hemoglobin or in units per volume of packed or suspended cells, and in doing so, they are referencing their measurements to parameters which are not constant with either donor or cell age.

Also, Thienhaus et al. (1986) measured CuZnSOD activity in skin fibroblasts from a 53-year-old male patient with familial Alzheimer's disease (his pedigree contained multiple cases of diagnosed Alzheimer's disease) and compared the enzyme activity to a 14-year-old female Down's syndrome patient and a normal, healthy 55-year-old man who served as a control. SOD activity was measured in units per protein content in the assay tube. These investigators found that the CuZnSOD activity was significantly higher in fibroblasts obtained from the Alzheimer's disease patient compared to that observed in normal euploid fibroblasts. Nevertheless, it is difficult to extrapolate from these results since such a small sample size (i.e., 3 subjects) was used which could conceivably bias the results.

Similarly, Zemlan et al. (1988) employed fibroblast cell lines derived from 8 familial Alzheimer's disease patients (5 females and 3 males; mean age = 60.6 ± 8.7 years), 12 normal individuals (7 females and 5 males; mean age = 62.2 ± 11.0 years), and 3 trisomy 21 patients (a 13-year-old girl, a 14-year-old girl, and a 37-year-old woman). CuZnSOD activity was measured in international units per million cells (a Coulter counter was used to count cells). These researchers found a significant 30% enhancement in CuZnSOD activity in Alzheimer's

disease patients when compared to normal controls. The Down's syndrome patients showed an even more dramatic 42% increase in CuZnSOD activity when compared to the controls. This value was clearly significantly greater than that observed in the Alzheimer's disease patients.

On the other hand, Zubenko et al. (1989), in an extensive study, examined red blood cell SOD activity in 20 probable Alzheimer's disease patients (10 females and 10 males; mean age = 71.5 $\pm$ 6.8 years), 20 neurologically healthy age- and gender-matched controls (10 females and 10 males; 67.6 $\pm$ 5.8 years), and 20 healthy young controls (10 females and 10 males; mean age = 26.1 $\pm$ 4.5 years). These investigators reported CuZnSOD activity in units per gram hemoglobin content in the blood samples. As already mentioned, this weakens the significance of the findings since hemoglobin levels change with age and; therefore, may confound any alterations occurring in SOD activity when SOD activity is referenced with respect to hemoglobin content. The results of the study indicated a modest, but statistically significant age-related increase in enzyme activity in women only, while a modest, statistically significant gender difference was observed in the elderly control group where the elderly females exhibited higher CuZnSOD activity. The researchers suggested that the apparent sex difference in the relationship of age and red blood cell superoxide dismutase activity may be due to the fact that the younger group was pre-menopausal whereas the older group was post-menopausal. In addition, the investigators found no significant alteration in red blood cell CuZnSOD activity between Alzheimer's disease patients and the age-matched control subjects.

Furthermore, Sulkava et al. (1986) examined CuZnSOD activity in four patients with non-familial Alzheimer's disease (3 females and 1 male; age range 58-74 years; mean age = 69.0 $\pm$ 7.4 years, 4 patients with multi-infarct dementia (3 females and 1 male; age range 57-83 years;

mean age = 72.2 $\pm$ 11.0 years), 1 Huntington's disease patient (female; 33-years-old), and one patient with Hakola-Nasu disease (female; 44-years-old). Control blood samples were obtained from healthy medical staff members (3 females and 2 males, age range 48-64 years; mean age = 54.8 $\pm$ 7.9 years). CuZnSOD activity was reported in units per gram hemoglobin. The results of this study revealed no significant differences in erythrocyte SOD activity in the 10 patients with different types of dementia and in the controls. The SOD activity, however, showed an age-related decline in the control subjects. Among the demented patients, there was no correlation between enzyme activity and age. A problem with this study is that the researchers examined only a small series of patients; therefore, chance may be affecting the results. In addition, age-matched controls were not used which may influence the findings. Alzheimer's disease patients and multi-infarct patients were on average 15-18 years older than the controls.

A Final Word

Elucidation of the role of free radicals in pathogenesis of Alzheimer's disease might open new areas for development of prevention measures and/or treatment strategies. Preventive measures might be especially useful because there is an indication that, at least in some patients, there is a long subclinical phase of disease development (Tarvik, 1988). This approach, however, requires availability of a method by which individuals at risk for Alzheimer's disease, or at a subclinical stage of Alzheimer's disease, would be identified.

EXPERIMENTAL AIM AND DESIGN

HYPOTHESIS

A review of the literature indicates that theoretically, a relative excess or deficit of uZnSOD activity could be physiologically deleterious to individuals as either situation promotes the formation of hydroxyl radicals and leads to unscheduled oxidations of biological material. Thus, it was hypothesized that Alzheimer's disease patients will have significantly altered CuZnSOD activities in their red blood cells compared to age-matched neurologically healthy elderly controls.

PURPOSE

To investigate whether or not there are alterations in CuZnSOD activity levels in the red blood cells of Alzheimer's disease patients compared to neurologically healthy age-matched controls.

METHODS

Red blood cell CuZnSOD total activity, extract protein concentration, and hemoglobin levels were determined from Alzheimer's disease patients and age-matched neurologically healthy controls. Also, a measure of the energy of activation of CuZnSOD was determined by using the Arrhenius equation. CuZnSOD activity was reported as units of activity per gram of hemoglobin, per gram of total protein, and per milliliter of packed red blood cells so that comparisons can be made with literature values.

Subjects

Alzheimer patient's blood was obtained form the Alzheimer's clinic at the University of British Columbia Hospital. Blood from old neurologically healthy controls was collected from volunteers. Written informed consent was provided by all subjects before entry into the study. The Alzheimer's disease group met currently accepted clinical criteria for probable Alzheimer's disease. The Alzheimer's patients group consisted of 28 subjects (14 males; 14 females), with a mean age of 74.4 $\pm$ 7.8 years and an age range of 60.0 - 97.0 years. The control subjects group was comprised of 31 individuals (22 males; 9 females), with a mean age of 72.1 $\pm$ 8.4 years, and an age range of 60.7 - 86.8 years.

Blood Samples

A course was taken to learn how to withdraw and collect blood.

7.5 mL whole blood samples were collected in heparinized tubes by venipuncture to the antecubital vein in subjects.

Preparation of CuZnSOD Extract

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

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

(3) 0.5 mL of erythrocytes was 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 was removed by adding 3 mL of chloroform and 5 mL of ethanol according to the Tsuchihashi method. This step is critical as hemoglobin (Hb) interferes with the SOD assay by counteracting the inhibitory effect of SOD on cytochrome C reduction.

(5) The contents of the centrifuge tube were vigorously vortex-mixed for at least one minute and a thick white precipitate accumulated.

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

(7) The almost clear supernatant was diluted by a factor of 100 (This large dilution precluded the necessity for dialysis to remove low MW substances).

(8) Volumes ranging from 0 to 480 uL of the diluted solution were used to assay for CuZnSOD activity.

Superoxide Dismutase Assay

The L'Abbe and Fischer method (a xanthine/xanthine oxidase/cytochrome C indirect assay of SOD; a modification of the McCord and Fridovich method, 1969) was used to assay CuZnSOD activity with minor alterations. The pH was increased from pH 7.8 to pH 10.0 since the assay for CuZnSOD is approximately 17 times more sensitive at this higher pH (L'Abbe and Fischer, 1986). Furthermore, the MnSOD, which is inhibited at this pH, does not interfere with the assay when it is performed at this pH (L'Abbe and Fischer, 1986). The samples were extracted with chloroform and ethanol since it removes the hemoglobin and destroys the MnSOD, but leaves the CuZnSOD activity intact (L'Abbe and Fischer, 1986). The reduction of

cytochrome C was measured at the more sensitive 415 nm wavelength compared to the 550 nm wavelength used in most assays.

Materials Used in Assay of CuZnSOD:

Reagents:

* sodium carbonate buffer

* ferricytochrome C (type VI from horse heart, SIGMA)

* xanthine oxidase (xanthine: oxygen oxidoreductase, Grade I from buttermilk, SIGMA)

Note: 20 uL of xanthine oxidase was added to the cuvette during the assay

*CuZnSOD (SIGMA)

Stock Solutions:

* 20 mM sodium carbonate buffer, pH 10.0 containing 0.1 mM EDTA

* 500 uM ferricytochrome C (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 cytochrome C solution

* 5 mL xanthine solution

Note: This mixture is stable for several weeks when kept in the refrigerator. Since it contains cytochrome C, it must be covered with foil to prevent light penetration.

SOD Curve:

A 1/percentage inhibition versus 1/uL SOD extract curve was plotted for each subject. Superoxide is generated by the following reaction:

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

This reaction was monitored at 415 nm on a Hewlett Packard spectrophotometer as an increase in absorbance due to the reduction of cytochrome C.

SOD, which catalyzes the dismutation of superoxide ($2O_2^{\cdot-} \rightarrow H_2O_2 + O_2$), inhibits the inhibits the reduction of cytochrome C.

Percentage Inhibition:

Percentage inhibition = (control rate-sample rate)/control rate X 100%

Unit SOD Activity:

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

Therefore, the equation

$$\frac{1}{\%I} = \frac{Km}{\%I\ max} \cdot \frac{1}{[SOD]} + \frac{1}{\%I\ max}$$

where %I = 50%

%I max = 100%

Km/%I max = slope of the line (for 1/% I vs 1/uL SOD extract)

1/[SOD] = unit/uL

can be used to calculate one unit of activity in the cuvette.

Activity Calculation: Units SOD/mL packed RBCs = (1000 uL)/(uL/unit) x 1000 where multiplying by 1000 uL corrects for the dilution in the cuvette and multiplying by 1000 corrects for the dilution of the extract to the red blood cell.

Protein Determination

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

The Drabkin method for hemoglobin determination was performed on the blood samples (Drabkin and Austin, 1935).

SDS Gel Electrophoresis

Samples of the SOD extract were analyzed by polyacrylamide gel electrophoresis. SDS slab mini-gels were run in 5% acrylamide. Shortly after the gels were developed, the pattern of bands was sketched. Two lanes containing standards: glycogen phosphorylase (100 kDa), serum albumin (67 kDa), and superoxide dismutase (16 kDa) were used. Distance of migration of the known bands were plotted versus log molecular weight. From this curve, molecular weight values for the unknown proteins surviving the chloroform/ethanol extraction procedure were estimated.

Enzyme Kinetic Analysis

A graph of percent inhibition versus uL of CuZnSOD gives a hyperbolic curve. However, transforming this plot into a plot of 1/percent inhibition versus 1/uL of CuZnSOD gives a linear curve which is analogous to the Lineweaver-Burke equation. Also, the parameters obtained are similar to the Lineweaver-Burke parameters.

$$\%I = \frac{\%I\ max\ [SOD]}{Km + [SOD]} \text{ is analogous to } v = \frac{Vm\ [S]}{Km + [S]}$$

$$\frac{1}{\%\ I} = \frac{Km}{\%I\ max} \cdot \frac{1}{[SOD]} + \frac{1}{\%I\ max} \text{ is analogous to } \frac{1}{v} = \frac{Km}{Vm} \cdot \frac{1}{[S]} + \frac{1}{Vm}$$

where %I max = 100% inhibition.

The slope of this graph is equal to $\frac{Km}{\%\ I\ max}$ which is analogous to $\frac{Km}{Vm} = \frac{Km}{kcat\ Et}$.

Therefore, 1/slope approximately equals $\frac{kcat\ Et}{Km}$,

where kcat = a physical property of the enzyme

Et = amount of enzyme present (a reflection of rate of enzyme synthesis and/or degradation)

Km = concentration of substrate which gives half maximal velocity

= concentration of SOD which gives 50% inhibition of the xanthine oxidase reaction

= 1 unit of SOD activity

1/slope = relative SOD activity

Note: An increase in kcat and/or Et will both increase the 1/slope value.

The slope of a plot of 1/slope (kcat Et/Km)' versus extract Lowry (or Bradford) protein can be affected by changes in kcat, Et, and/or differences in the amount of total protein surviving in the extracts.

Arrhenius Plot

Arrhenius Equation: $\ln kcat = -\frac{Ea}{RT} + \ln A = -\frac{Ea}{R} \cdot \frac{1}{T} + \ln A$

This is qualitatively the same as $\ln \% I = -Ea' \cdot \frac{1}{T} + \log A$

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

Note: Cytochrome C and CuZnSOD compete for capture of the superoxide radical at an increased rate as a function of temperature. Therefore, the assay actually measures the relative ability of control subject's CuZnSOD and Alzheimer's disease patient's CuZnSOD to compete with cytochrome C. Since the cytochrome C used was the same in all subjects, any factors which cause cytochrome C rates to increase will be the same in the controls and the Alzheimer's disease subjects. Because this method is a modification of the classical Arrhenius plot, it is not possible to calculate activation energy (Ea) in kcal/mole. Nevertheless, the assay qualitatively reflects any changes in Ea or kcat/Km, which can result from any modification of the physical properties and catalytic efficiency of the enzyme.

Statistical Analysis

The SAS Statistical Program was utilized to perform an ANOVA (analysis of variance) in order to determine whether there were statistical differences ($p<0.05$) in mean values for (kcat Et/Km)', hemoglobin level, extract Lowry protein, extract Bradford protein, and Arrhenius slope between the subject groups. Also a regression analysis was performed to derive an equation which related (kcat Et/Km)' as a function of other influencing factors. Means and standard deviations were calculated for all experimental parameters. Also, the slope of the double

reciprocal plot of 1/% I versus 1/uL SOD graph was calculated two different ways. First, by simply plotting the data points and determining the best fit line. Second, by setting the y-intercept at 0.01 and then determining the slope of the best fit line. The second method removes the maximal value of %I as an unknown and replaces it with the known value of 100%. Standard deviations on the slope and y-intercept values were calculated for enzyme kinetic data using the SAS statistical package. The corrected values showed a strong agreement with values obtained from the original double reciprocal plot when the y-intercept was not forced. Thus, in all calculations, comparisons, and plots, the raw value for slope was used.

RESULTS

Blood specimens from Alzheimer's patients and neurologically healthy, age-matched controls were analyzed in this study. A comparison of the ages of the Alzheimer's patients and the control subjects revealed that there was no significant difference between the ages of these two subject groups (Figure 1). Thus, any changes observed between the two subject populations were not a result of differences in the ages of these groups. CuZnSOD activities were compared between males and females; however, no significant differences were elucidated based on the sex of the subjects.

The blood samples from each patient were extracted in chloroform/ethanol using the McCord and Fridovich method (1969) and CuZnSOD was measured by the L'Abbe and Fischer method (1986). The SOD chloroform/ethanol extracts were used to measure relative SOD activity, the total protein surviving the chloroform/ethanol extraction as determined by the Lowry method and Bradford method, and the relative energy of activation (Ea'). Table 1 shows a

summary of data of all experimental parameters obtained for each subject. Volumes ranging from 0 to 480 uL of chloroform/ethanol extract were used to assay SOD activity via an indirect method using an inhibition reaction where the CuZnSOD enzyme competitively inhibits the reduction of cytochrome C by the superoxide radical. A plot of (1/% inhibition) versus (1/uL from the aqueous phase of the chloroform/ethanol extract) was graphed and its slope provided a value which is a reflection of SOD activity. A summary of the graphs obtained for the Alzheimer's patients (Figure 2) and for the control subjects (Figure 3) is presented. The inverse of this slope is proportional to (kcat Et/Km)'. This value (kcat Et/Km)' was plotted against various parameters including: protein surviving the chloroform/ethanol extract as determined by the Lowry method and the Bradford method, and Drabkin hemoglobin values. Table 2 contains a summary of all of the slopes as well as the slopes corrected for the y-intercept for all of the subjects. In addition, standard deviation of the slopes and the y-intercepts are also given (Table 2).

A bar graph (Figure 4) of relative superoxide dismutase activities (kcat Et/Km)' in erythrocyte extracts from Alzheimer's patients and control subjects gave a mean value of 1.05 $\pm$ 0.42 for the Alzheimer's patients and 0.48 $\pm$ 0.22 for the control subjects. This figure indicates that the Alzheimer's patients had significantly more (approximately twice) the CuZnSOD activity in their erythrocytes compared to the control subjects ($p<0.05$).

Total SOD activity (i.e., Units SOD activity/mL packed red blood cells) in the blood was calculated from the value of (kcat Et/Km)' by multiplying this value be 10 000, which takes into account the dilution factors from the red blood cells. Thus, the correlation between these two values should be perfect. A plot of SOD activity (in Units/mL packed RBCs) against relative

SOD activity (kcat Et/Km)' gives the expected perfect correlation (r = 1.0) for these two parameters (since they differ by a constant -- the dilution factor to the blood) for both the Alzheimer's patients (Figure 5) and the control subjects (Figure 6). Therefore, relative SOD activity (kcat Et/Km)' is directly proportional to activity given in Units SOD/mL packed red blood cells and it can be substituted for (kcat Et/Km)'. These two values can be used interchangeably. Any relationship which exists for one will hold for the other with respect to other variables.

A bar graph of SOD activity (Units/mL packed red blood cells) from Alzheimer's patients and control subjects gave a mean magnitude of 10 798 and 4 881, respectively (Figure 7). Thus, on average, the Alzheimer's patients have approximately twice the CuZnSOD activity present in the age-matched controls.

The graph of relative SOD activity (kcat Et/Km)' in the extracts from subjects versus total protein surviving the chloroform/ethanol extraction as measured by the Lowry method is a linear relationship and shows a positive correlation for both subject groups. The magnitude of the slope of this graph is 0.77 (r = 0.84) for the Alzheimer's patients (Figure 8A) compared to the slope of 0.085 (r = 0.85) for the age-matched, neurologically healthy controls (Figure 8B). The Alzheimer's patients, thus, have a slope for this graph which is approximately ten times steeper than the old healthy controls. This finding shows that (kcat Et/Km)'/Lowry protein is significantly higher for the Alzheimer's patients compared to the controls. A plot of relative SOD activity (kcat Et/Km)' versus extract Lowry protein (ug/mL) was made showing the results for both Alzheimer's patients and control subjects on the same graph (Figure 9) to better show the differences between these groups.

Similarly, a plot of the relationship between relative SOD activity in the red blood cell extract from the Alzheimer's patients and control subjects compared to the amount of total protein surviving the chloroform/ethanol extraction procedure as determined by the Bradford method gave a linear graph with a positive correlation for the Alzheimer's patients (Figure 10) and the control subjects (Figure 11). The best-fit curve through the data points gives a slope of 0.84 ($r = 0.82$) for the Alzheimer's patients (figure 10) and a slope of 0.084 ($r = 0.81$) for the control subjects (figure 11). These results also indicate that the ratio (kcat Et/Km)'/protein in the chloroform/ethanol extracts is significantly greater for the Alzheimer's patients compared to the controls.

The relation of relative SOD activity (kcat Et/Km)' per extract Lowry protein in Alzheimer's patients and control subjects was graphed (Figure 12). The Alzheimer's patients had a value of 0.857 for this parameter compared to 0.096 in the control subjects. This graph reveals that the value of the ratio of (kcat Et/Km)'/extract Lowry protein in the Alzheimer's patients is approximately ten times higher than that obtained for the control subjects.

Another bar graph demonstrating the relationship between relative SOD activity (kcat Et/Km)' per extract Bradford protein in Alzheimer's patients and control subjects was also made (Figure 13). The Alzheimer's patients had a value of 1.10 for this ratio while the control subjects had a value of 9.90 for this same ratio. The interpretation for this graph is the same as that for the Lowry graph described above. SOD activity is proportional to the protein content in the aqueous phase of the chloroform/ethanol extracts within each group. This is expected since the relative efficiency of the SOD purification should be proportional to the purification of those proteins capable of surviving the chloroform/ethanol extraction procedure (Fridovich, personal

communications).

A bar graph (Figure 14) of the relative amounts of Lowry protein surviving the chloroform/ethanol extraction of erythrocytes indicates that the Alzheimer's patients had 1.22 $\pm$ 0.46 ug/mL of protein in the extract while the control subjects had 5.34 $\pm$ 2.49 ug/mL of protein in the extract. A bar plot (Figure 15) of the relative amounts of Bradford protein surviving the chloroform/ethanol extraction of erythrocytes from Alzheimer's patients had 1.10 $\pm$ 0.41 ug/mL of protein in their extracts; the controls had 4.62 $\pm$ 2.26 ug/mL of protein in their extracts. Therefore, blood samples from the Alzheimer's patients compared to blood specimens from the control subjects show a significant difference ($p<0.05$) in the ability of their proteins to survive the harsh chloroform/ethanol treatment. It appears that the Alzheimer's patients have approximately 4-5 times less the amount of total protein surviving the chloroform/ethanol extraction compared to the age-matched controls.

A comparison between the values estimated for protein in the chloroform/ethanol extract of red blood cells obtained by the Bradford protein determination method versus the Lowry protein determination method was conducted (Figure 16). The equation for the best-fit curve through these data points is $y = -0.011 + 0.88x$ ($r = 0.99$). Thus, there was a very good correlation between the Lowry method and the Bradford method for determining the protein concentration in the extracts from the subjects; however, Bradford values were consistently lower (approximately 88% of the Lowry values) than protein measurements obtained using the Lowry method. The Lowry method has been documented to pick up glutathione in the preparations as it is sensitive to reducing agents such as cysteine. The Bradford method cannot measure small peptides.

A plot of Units SOD activity/mL packed red blood cells versus extract Lowry protein was graphed for each subject class. The graph was linear, with a positive correlation, for both Alzheimer's patients (Figure 17) and control subjects (Figure 18). The line delineating the relationship between these two variables is $y = 992 + 7\ 671x$ ($r = 0.84$) for the Alzheimer's patients (Figure 17) and $y = 775 + 760x$ ($r = 0.85$) for the control subjects (Figure 18). This shows that the slopes are about ten times higher in the Alzheimer's patients compared to the control subjects.

A similar plot of Units SOD activity/mL packed red blood cells versus the extract proteins detected by the Bradford protein determination method was also made. The function defining this graph is $y = 1130 + 8\ 449x$ ($r = 0.82$) for the Alzheimer's patients (Figure 19) and $y = 1284 + 754x$ ($r = 0.78$) for the control subjects (Figure 20). Thus, the ratio of Units SOD/mL packed red blood cells to total protein contained in the chloroform/ethanol extract is much greater for the Alzheimer's patients compared to the control subjects.

Results from the SDS-PAGE mini-slab gel (Figure 21) reveals four bands appearing in the lanes which contain chloroform/ethanol extract from the Alzheimer's patients and the control subjects. These bands have been tentatively assigned as glutathione reductase (62 kDa), catalase (54 kDa), CuZnSOD and hemoglobin (16 kDa), and calmodulin (14 kDa). Therefore, the chloroform/ethanol purification procedure removed most of the red blood cell proteins as only five proteins were detected on the gel. Only very hardy proteins can survive this extraction procedure. The relative intensity of the 54 kDa band to the 16 kDa band was considerably lower in the Alzheimer's patients compared to the control subjects.

A graph of SOD units/mL packed red blood cells as a function of hemoglobin content in

the Alzheimer's patients (Figure 22) and the control subjects (Figure 23) revealed a scatter plot for both the Alzheimer's patients ($y = 15\ 259 - 321x$; $r = 0.14$) and controls ($y = 5\ 755 - 55x$; $r = 0.05$). Note, the computer program used draws a line to best-fit the data points; nevertheless, the r value is very low indicating that no correlation exists. Thus, no correlation exists between SOD activity and hemoglobin content. A bar plot of hemoglobin levels determined by the Drabkin method in erythrocytes from Alzheimer's patients and control subjects (Figure 24) gave values of 15.4 ± 2.5 and 16.0 ± 2.2, respectively. No significant difference exists between hemoglobin levels in the Alzheimer's patients compared to the control subjects.

A graph of Units of SOD/mL packed red blood cells/g of hemoglobin was plotted as a function of extracted Lowry protein for both the Alzheimer's patients (Figure 25) and the control subjects (Figure 26). The linear equation fitting this graph is $y = 50 + 541x$ ($r = 0.75$) for the Alzheimer's patients and $y = 62 + 46x$ ($r = 0.77$) for the control subjects. The differences observed in the slopes of these two graphs is a reflection of differences in both the SOD activities between the two subject groups as well as differences in the ability of proteins from the two subject classes to withstand the chloroform/ethanol extraction procedure.

A graph of Units of SOD activity/mL packed red blood cells/g of hemoglobin as a function of extracted Bradford protein was also plotted for Alzheimer's patients (Figure 27) and control subjects (Figure 28). The line defining this function is $y = 104 + 540x$ ($r = 0.74$) for the Alzheimer's patients and $y = 97 + 45x$ ($r = 0.69$) for the control subjects. The interpretation of this graph is similar to that given for the graph described above.

Units SOD activity per mL packed red blood cells per gram of hemoglobin in erythrocytes from Alzheimer's patients and control subjects was expressed as a bar graph (Figure 29) and

gave a value of 702 units/mL packed red blood cells/g Hb for the Alzheimer's patients compared to 311 Units/mL packed red blood cells/g Hb in the controls. This graph demonstrates that Alzheimer's patients have approximately twice the amount of units of SOD activity per mL red blood cells per gram of hemoglobin compared to the control subjects.

In addition, a bar graph of the relative Arrhenius (Temp.) slopes (derived from a graph of ln % inhibition versus 1/Temperature) for SOD in Alzheimer's patients and control subjects was plotted against the subject classes (Figure 30). The magnitude of this value for the Alzheimer's patients was -2159.6 ± 4731.4 and for the control subjects it was -451.5 ± 1173.3. Statistical analysis revealed that a 90% probability exists ($p<0.1$) for significant differences in the means of the relative Arrhenius slopes between the Alzheimer's patients and the control subjects. Therefore, the Alzheimer's patient's SOD exhibited a higher energy of activation compared to the controls (since the Arrhenius slope = $-E_a$).

A summary of the means and standard deviations for important experimental parameters used in this study is given for the Alzheimer's patients (Table 3) and the control subjects (Table 4). Output from the SAS statistical program showing Pearson correlations for these parameters is exhibited in Table 5. A summary of statistical correlations ($p<0.05$) which exist between the various experimental parameters are given for the Alzheimer's patients (Table 6) and for the control subjects (Table 7). Output from the SAS program which calculates variance in the experimental values between the subject classes is given in Table 8. A summary showing experimental parameters whose variance is statistically significant is indicated in Table 9.

Table 1 Summary of Experimental Parameters

Subject	Age (yrs)	Hb (g/dL)	Lowry (ug/mL)	Bradford (ug/mL)	(kcatEt/Km)'/Lowry	(katEt/Km)'/Bradford	1/slope (kcatEt/Km)'	Units SOD/mL RBC	Units SOD/mL RBC/g
A1	63.000		1.415	1.076					
A2	71.000		2.000	1.631	0.728	1.120	1.456	14548.98	
A3	80.000		2.223	1.847	0.803	1.035	1.786	17772.51	
A4	75.570	13.976	1.099	0.985	0.726	1.134	0.798	7944.92	568
A5	79.000	16.192						5131.71	317
A6	80.000	16.088	0.814	0.827	0.722	1.406	0.588	5875.44	365
A7	71.000	15.936	0.678	0.525	0.987	0.784	0.670	6705.41	421
A8	66.000	14.624	0.918	0.905	1.371	0.719	1.259	12594.46	861
A9	72.000	14.304						7507.51	525
A10	77.000	14.288	0.905	0.867	0.690	1.387	0.625	6242.20	437
A11	76.000	17.152	1.143	1.102	0.663	1.455	0.758	7583.42	442
A12	76.000	18.104	1.982	1.568	0.694	1.140	1.376	13799.45	762
A13	67.000	14.496	0.948	0.648	1.014	0.674	0.962	9584.66	661
A14	67.000	15.248	1.174	1.092	0.933	0.997	1.095	10972.93	7E+2
A15	78.000	12.992						26833.63	2065
A16	71.000	15.288	1.598	1.852	0.939	1.235	1.500	15060.24	985
A17	84.000	11.248	1.671	1.532	0.926	0.990	1.546	15463.92	137
A18	68.000	16.640		1.222		0.937	1.304	12998.27	781
A19	64.000	17.176	0.529	0.437	0.699	1.183	0.369	3690.04	215
A20	84.000	14.216	0.942	0.776	1.083	0.761	1.020	10204.08	718
A21	97.000	11.088	1.358	1.268	0.566	1.649	0.769	7708.12	695
A22	72.000	13.096	1.347	1.296	0.718	1.339	0.968	9683.67	739
A23	76.000	18.256	1.807	1.625	1.137	0.791	2.055	20519.84	1124
A24	81.000	11.288	1.015	0.928	0.890	1.040	0.893	9907.36	789
A25	60.000	17.400	0.724	0.579					
A26	77.000	17.776	1.145	1.110	0.712	1.362	0.815	8130.08	457
A27	69.000	18.992					0.761	7625.83	402
A28	82.000	18.128	0.755	0.749	1.013	0.979	0.765	7672.63	423
C1	60.700	15.818	3.885	3.483	0.114	7.889	0.442	4415.01	279
C2	60.860								
C3	60.860	21.900	5.248	4.394	0.061	13.692	0.321	3209.24	146
C4	63.000	16.223	11.211	9.566				9621.55	593
C5	63.440	12.585	3.646	3.545	0.091	10.639	0.333	3331.85	265
C6	63.790	17.745	5.737	5.312	0.093	9.903	0.536	5362.89	302
C7	63.880	17.460	3.260	2.034	0.153	4.068	0.500	5000.00	286
C8	64.320	16.343	4.094	3.128	0.067	11.365	0.275	2747.11	168
C9	65.150	18.285	4.526	4.440	0.071	13.881	0.320	3198.98	175
C10	66.000	16.395	5.381	4.527	0.069	12.232	0.370	3700.96	226
C11	67.000	17.280	3.030		0.100		0.303	3029.48	175
C12	67.000	15.113	3.890	3.563	0.065	14.255	0.251	2513.57	166
C13	68.110	15.713	5.538	5.935	0.096	11.212	0.529	5292.87	337
C14	68.160	14.955	6.608	5.582	0.067	12.566	0.444	4441.81	297
C15	68.930	14.940	3.832	3.501	0.139	6.591	0.531	5311.61	356
C16	69.790	18.353	7.678		0.107		0.819	8187.77	446
C17	70.600	13.260	3.269	1.842	0.130	4.345	0.424	4239.68	320
C18	71.380	17.265	10.763	9.860	0.106	8.617	1.144		
C19	72.580	14.723		5.158		8.022	0.643	6429.49	437
C20	76.980	19.935	7.521	6.321	0.078	10.741	0.589	5894.66	295
C21	79.040	15.293	10.775	9.804	0.079	11.515	0.851	8513.05	557
C22	79.000	13.260	4.175	4.421	0.069	15.366	0.298	2876.87	217
C23	80.000	16.268	4.217	3.546	0.093	9.027	0.393	3928.76	242
C24	80.000	14.565	8.385	7.004	0.105	7.966	0.879	8792.50	604
C25	80.880	16.073	3.840	3.804	0.071	14.131	0.271	2712.97	169
C26	81.000	12.750	3.286	2.903	0.115	7.662	0.379		
C27	82.000	14.723	3.557	4.166	0.152	7.702	0.541	5409.30	367
C28	84.000	12.518	3.330	2.061	0.124	4.986	0.413	4133.37	330
C29	84.000	16.553	2.386	2.079	0.101	8.609	0.241		
C30	85.880	13.950	7.825					6793.48	487
C31	86.830	18.518	3.865	2.852	0.070	10.547	0.270	2703.68	146

Table 1 Summary of Experimental Parameters

Subject	Age (yrs)	Hb (g/dL)	Lowry (ug/mL)	Bradford (ug/mL)	(kcatEt/Km)'/Lowry	(katEt/Km)'/Bradford	1/slope (kcatEt/Km)'	Units SOD/mL RBC	Units SOD/mL RBC/g Hb
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A2	71.000		2.000	1.631	0.728	1.120	1.456	14548.98	
A3	80.000		2.223	1.847	0.803	1.035	1.786	17772.51	
A4	75.570	13.976	1.099	0.905	0.726	1.134	0.798	7944.92	568
A5	79.000	16.192						5131.71	317
A6	80.000	16.088	0.814	0.827	0.722	1.406	0.588	5875.44	365
A7	71.000	15.936	0.678	0.525	0.987	0.784	0.670	6705.41	421
A8	66.000	14.624	0.918	0.905	1.371	0.719	1.259	12594.46	861
A9	72.000	14.304						7507.51	525
A10	77.000	14.288	0.905	0.867	0.690	1.387	0.625	6242.20	437
A11	76.000	17.152	1.143	1.102	0.663	1.455	0.758	7583.42	442
A12	76.000	18.104	1.982	1.568	0.694	1.140	1.376	13799.45	762
A13	67.000	14.496	0.948	0.648	1.014	0.674	0.962	9584.66	661
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A19	64.000	17.176	0.529	0.437	0.699	1.183	0.369	3690.04	215
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A25	60.000	17.400	0.724	0.579					
A26	77.000	17.776	1.145	1.110	0.712	1.362	0.815	8130.08	457
A27	69.000	18.992					0.761	7625.83	402
A28	82.000	18.128	0.755	0.749	1.013	0.979	0.765	7672.63	423
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C8	64.320	16.343	4.094	3.128	0.067	11.385	0.275	2747.11	168
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C22	79.000	13.260	4.175	4.421	0.069	15.366	0.288	2876.87	217
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C24	80.000	14.565	8.385	7.004	0.105	7.966	0.879	8792.50	604
C25	80.880	16.073	3.840	3.804	0.071	14.131	0.271	2712.97	169
C26	81.000	12.750	3.286	2.903	0.115	7.662	0.379		
C27	82.000	14.723	3.557	4.166	0.152	7.702	0.541	5409.30	367
C28	84.000	12.518	3.330	2.064	0.124	4.986	0.413	4133.37	330
C29	84.000	16.553	2.386	2.079	0.108	8.609	0.341		
C30	85.880	13.950	7.825					6793.48	487
C31	86.830	18.518	3.865	2.852	0.070	10.547	0.270	2703.68	146

Table 1 Summary of Experimental Parameters (cont.)

Temp. slope	Subject Group
-3454.432	Alzheimer's
-413.583	Alzheimer's
	Alzheimer's
-4099.743	Alzheimer's
-1204.238	Alzheimer's
-401.399	Alzheimer's
-777.737	Alzheimer's
	Alzheimer's
-21487.775	Alzheimer's
	Alzheimer's
-118.306	Alzheimer's
-290.213	Alzheimer's
-83.886	Alzheimer's
-446.687	Alzheimer's
-473.003	Alzheimer's
672.307	Alzheimer's
-1364.385	Alzheimer's
-97.349	Alzheimer's
-1080.330	Alzheimer's
-340.291	Alzheimer's
1145.988	Alzheimer's
-1257.877	Alzheimer's
-1402.264	Alzheimer's
	Alzheimer's
-1406.344	Alzheimer's
-2257.929	Alzheimer's
-720.347	Alzheimer's
135.120	Controls
	Controls
843.380	Controls
-784.879	Controls
-2299.274	Controls
-346.632	Controls
82.334	Controls
1190.944	Controls
347.886	Controls
247.299	Controls
	Controls
	Controls
-479.064	Controls
	Controls
-402.080	Controls
-780.294	Controls
235.279	Controls
-47.028	Controls
-332.442	Controls
-398.069	Controls
330.426	Controls
2005.837	Controls
	Controls
-348.973	Controls
-1149.307	Controls
-448.397	Controls
	Controls
	Controls
-2252.242	Controls
-2231.466	Controls
-2104.033	Controls

Table 2 Summary of raw data and sas corrected values for slope and y-intercept of 1/% inhibition vs 1/uL SOD plots

sample	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 SD	corrected slope T value	corrected slope P value	Dilution Factor
A1	0.012	0.0011	10.700	0.00010	1.268	0.101	12.600	0.00010	1.394	0.068	20.500	0.00010	1.5
A2	0.011	0.0009	12.100	0.00010	1.031	0.073	14.100	0.00010	1.094	0.048	22.900	0.00010	1.5
A3	0.011	0.0092	12.200	0.00010	0.844	0.077	11.000	0.00010	0.922	0.048	19.100	0.00010	1.5
A4	0.010	0.0010	10.500	0.00050	1.888	0.111	17.100	0.00010	1.896	0.049	38.800	0.00010	1.5
A5	0.010	0.0018	5.700	0.00040	2.923	0.139	21.100	0.00010	2.950	0.081	36.200	0.00010	1.5
A6	0.010	0.0016	6.200	0.00030	2.553	0.123	20.700	0.00010	2.554	0.072	35.500	0.00010	1.5
A7	0.010	0.0015	6.800	0.00010	2.237	0.111	20.100	0.00010	2.236	0.065	34.300	0.00010	1.5
A8	0.010	0.0006	17.200	0.00010	1.191	0.041	29.300	0.00010	1.205	0.023	51.100	0.00010	1.5
A9	0.010	0.0012	8.300	0.00010	1.998	0.094	21.300	0.00010	2.010	0.055	36.500	0.00010	1.5
A10	0.011	0.0020	5.200	0.00050	2.403	0.153	15.700	0.00010	2.434	0.087	28.000	0.00010	1.5
A11	0.010	0.0010	9.800	0.00010	1.978	0.075	26.500	0.00010	1.986	0.041	48.400	0.00010	1.5
A12	0.010	0.0006	17.300	0.00010	1.087	0.043	25.100	0.00010	1.077	0.025	42.300	0.00010	1.5
A13	0.011	0.0089	12.100	0.00010	1.565	0.080	19.600	0.00010	1.618	0.051	32.000	0.00010	1.5
A14	0.010	0.0005	20.200	0.00010	1.367	0.044	30.800	0.00010	1.385	0.028	49.000	0.00010	1.5
A15	0.010	0.0004	23.500	0.00010	0.559	0.033	16.900	0.00010	0.571	0.020	29.200	0.00010	1.5
A16	0.010	0.0006	15.700	0.00010	0.996	0.052	19.200	0.00010	0.982	0.031	31.300	0.00010	1.5
A17	0.010	0.0006	16.900	0.00010	0.970	0.048	20.400	0.00010	0.977	0.029	34.000	0.00010	1.5
A18	0.011	0.0010	10.300	0.00010	1.154	0.079	14.700	0.00010	1.193	0.047	25.300	0.00010	1.5
A19	0.001	0.0020	5.200	0.00670	4.065	0.370	11.000	0.00040	4.088	0.154	26.500	0.00010	1.5
A20	0.010	0.0005	17.800	0.00010	1.470	0.041	35.600	0.00010	1.453	0.026	57.000	0.00010	1.5
A21	0.010	0.0013	7.500	0.00010	1.946	0.101	19.200	0.00010	1.949	0.059	32.900	0.00010	1.5
A22	0.010	0.0006	15.900	0.00010	1.549	0.047	32.900	0.00010	1.540	0.028	55.700	0.00010	1.5
A23	0.010	0.0009	11.500	0.00010	0.731	0.067	10.900	0.00010	0.736	0.039	18.800	0.00010	1.5
A24	0.010	0.0008	12.500	0.00010	1.664	0.060	28.100	0.00010	1.677	0.035	47.800	0.00010	1.5
A25	0.010	0.0079	12.500	0.00010	1.733	0.060	29.000	0.00010	1.721	0.035	49.000	0.00010	1.5
A26	0.010	0.0008	11.900	0.00010	1.845	0.064	29.100	0.00010	1.843	0.037	49.600	0.00010	1.5
A27	0.010	0.0010	10.100	0.00010	1.967	0.079	25.100	0.00010	1.994	0.047	42.900	0.00010	1.5
A28	0.010	0.0008	13.100	0.00010	1.955	0.059	33.000	0.00010	1.967	0.035	56.400	0.00010	1.5
C1	0.009	0.0013	7.140	0.00010	2.265	0.101	22.300	0.00010	2.212	0.063	35.400	0.00010	1.0
C3	0.011	0.0047	2.350	0.04670	3.116	0.357	8.740	0.00010	3.177	0.209	15.180	0.00010	1.0
C4	0.010	0.0010	9.700	0.00010	1.560	0.077	20.300	0.00010	1.540	0.044	35.400	0.00010	1.5
C5	0.010	0.0042	2.310	0.05000	4.502	0.318	14.100	0.00010	4.482	0.186	24.000	0.00010	1.5
C6	0.010	0.0035	2.780	0.02130	2.797	0.274	10.200	0.00010	2.772	0.165	16.800	0.00010	1.5
C7	0.010	0.0031	3.400	0.00850	3.000	0.246	12.200	0.00010	3.024	0.148	20.400	0.00010	1.5
C8	0.011	0.0034	3.240	0.01020	5.460	0.239	22.890	0.00010	5.520	0.141	39.040	0.00010	1.5
C9	0.010	0.0042	2.360	0.03750	4.689	0.344	13.600	0.00010	4.689	0.203	23.100	0.00010	1.5
C10	0.011	0.0032	3.550	0.00530	2.702	0.225	12.000	0.00010	2.779	0.131	21.300	0.00010	1.0
C11	0.011	0.0046	2.470	0.02930	3.30[illegible]	0.193	17.080	0.00010	3.344	0.123	27.230	0.00010	1.0
C12	0.010	0.0015	6.300	0.00040	3.978	0.151	26.300	0.00010	3.948	0.101	38.900	0.00010	1.0
C13	0.010	0.0075	7.100	0.00010	2.834	0.115	24.700	0.00010	2.855	0.067	42.700	0.00010	1.5
C14	0.010	0.0029	3.600	0.00450	3.377	0.202	16.700	0.00010	3.401	0.116	29.400	0.00010	1.5
C15	0.011	0.0036	3.100	0.01200	2.824	0.293	9.600	0.00010	2.889	0.175	16.500	0.00010	1.5
C16	0.010	0.0010	10.500	0.00010	1.832	0.073	25.200	0.00010	1.832	0.043	43.000	0.00010	1.5
C17	0.010	0.0030	3.250	0.01000	3.538	0.204	17.400	0.00010	3.522	0.117	30.100	0.00010	1.5
C18	0.012	0.0027	4.300	0.00260	1.311	0.207	6.300	0.00020	1.411	0.124	11.400	0.00010	1.5
C19	0.012	0.0013	8.900	0.00010	2.333	0.111	21.000	0.00010	2.458	0.071	34.700	0.00010	1.5
C20	0.010	0.0012	8.400	0.00010	2.546	0.091	27.900	0.00010	2.555	0.054	47.800	0.00010	1.5
C21	0.011	0.0008	12.900	0.00010	1.762	0.063	27.900	0.00010	1.801	0.037	48.900	0.00010	1.5
C22	0.011	0.0039	3.140	0.01640	5.214	0.467	11.160	0.00010	5.302	0.241	22.020	0.00010	1.5
C23	0.011	0.0022	4.800	0.00190	3.818	0.200	19.500	0.00010	3.855	0.114	33.700	0.00010	1.5
C24	0.011	0.0010	10.600	0.00010	1.706	0.075	22.700	0.00010	1.735	0.045	38.800	0.00010	1.5
C25	0.010	0.0027	3.600	0.00600	5.529	0.213	26.000	0.00010	5.511	0.123	44.600	0.00010	1.5
C26	0.011	0.0037	2.880	0.01800	3.959	0.253	15.700	0.00010	3.997	0.145	27.500	0.00010	1.5
C27	0.011	0.0007	17.000	0.00010	2.643	0.074	35.800	0.00010	2.756	0.045	61.700	0.00010	1.5
C28	-0.010	0.0048	2.180	0.06080	3.629	0.444	8.180	0.00010	3.663	0.253	14.480	0.00010	1.5
C29	0.012	0.0043	2.700	0.02700	4.140	0.325	12.720	0.00010	4.232	0.192	22.000	0.00010	1.0
C30	0.158	0.0992	1.590	0.21000	1.472	3.754	0.390	0.72100	5.580	2.907	1.920	0.12700	1.0
C31	0.010	0.0016	6.200	0.00030	5.542	0.123	45.000	0.00010	5.552	0.072	77.000	0.00010	1.0

Table 3 Summary of simple statistics on Alzheimer's patient's experimental parameters

	N	Mean	Standard Deviation	Sum	Minimum	Maximum
1/slope (kcatEt/Km)	23	1.05	0.42	24.14	0.37	2.05
Age (years)	28	74.41	7.84	2083.60	60.00	97.00
Hb (g/dL)	25	15.36	2.29	384.00	11.09	18.99
Lowry (ug/mL)	23	1.23	0.47	28.19	0.53	2.22
Bradford (ug/mL)	24	1.10	0.41	26.37	0.44	1.85
Temp. Slope	23	-2159.60	4731.42	-49670.50	-21487.80	1146.00

Table 4 Summary of simple statistics on control subject's experimental parameters

	N	Mean	Standard Deviation	Sum	Minimum	Maximum
1/slope (kcatEt/Km)	28	0.48	0.22	13.30	0.24	1.14
Age (years)	31	72.07	8.37	2234.20	60.70	86.83
Hb (g/dL)	30	15.96	2.19	478.80	12.52	21.90
Lowry (ug/mL)	29	5.34	2.49	154.80	2.39	11.21
Bradford (ug/mL)	27	4.63	2.27	124.90	1.84	9.86
Temp. Slope	24	-451.50	4731.42	-10835.90	-3104.00	2005.80

```
                              The SAS System                          1
                                          17:27 Monday, August 15, 1994

                     General Linear Models Procedure

                Number of observations in data set = 28


NOTE: Due to missing values, only 17 observations can be used in this
      analysis.

^L                               The SAS System                          2
                                          17:27 Monday, August 15, 1994

                     General Linear Models Procedure

Dependent Variable: INVSLOPE

Source                  DF     Sum of Squares      F Value       Pr > F

Model                    5         2.04528634         5.61       0.0083

Error                   11         0.80273460

Corrected Total         16         2.84802094

                  R-Square               C.V.            INVSLOPE Mean

                  0.718143           26.58269               1.01622705


Source                  DF          Type I SS      F Value       Pr > F

AGE                      1         0.00045392         0.01       0.9386
HB                       1         0.00769520         0.11       0.7515
L                        1         2.00907814        27.53       0.0003
B                        1         0.01062921         0.15       0.7100
ARHENIUS                 1         0.01742987         0.24       0.6346

Source                  DF        Type III SS      F Value       Pr > F

AGE                      1         0.09202642         1.26       0.2854
HB                       1         0.00025172         0.00       0.9542
L                        1         0.13387858         1.83       0.2028
B                        1         0.01299704         0.18       0.6811
ARHENIUS                 1         0.01742987         0.24       0.6346


                                T for H0:    Pr > |T|    Std Error of
Parameter          Estimate    Parameter=0                  Estimate

INTERCEPT      0.7893197782           0.82     0.4285      0.96013139
AGE            -.0102415666          -1.12     0.2854      0.00912010
HB             0.0018137695           0.06     0.9542      0.03088279
L              0.6666987131           1.35     0.2028      0.49222506
B              0.2089281034           0.42     0.6811      0.49506739
ARHENIUS       0.0000065919           0.49     0.6346      0.00001349
```

Table 5 Output from SAS Program on Pearson Correlations between experimental parameters

The SAS System 3
17:27 Monday, August 15, 1994

Correlation Analysis

6 'VAR' Variables: INVSLOPE AGE HB L B ARHENIUS

Simple Statistics

Variable	N	Mean	Std Dev	Sum	Minimum	Maximum
INVSLOPE	23	1.0495	0.4195	24.1391	0.3695	2.0548
AGE	28	74.4132	7.8355	2083.6	60.0000	97.0000
HB	25	15.3597	2.2889	384.0	11.0880	18.9920
L	23	1.2256	0.4655	28.1897	0.5285	2.2232
B	24	1.0987	0.4106	26.3687	0.4370	1.8520
ARHENIUS	23	-2159.6	4731.4	-49670.5	-21487.8	1146.0

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

	INVSLOPE	AGE	HB	L	B	ARHENIUS
INVSLOPE	1.00000	0.00407	0.00796	0.84214	0.82340	0.19461
	0.0	0.9853	0.9727	0.0001	0.0001	0.3979
	23	23	21	21	22	21
AGE	0.00407	1.00000	-0.45442	0.21760	0.26154	-0.03993
	0.9853	0.0	0.0225	0.3186	0.2170	0.8565
	23	28	25	23	24	23
HB	0.00796	-0.45442	1.00000	-0.08855	-0.09682	-0.01367
	0.9727	0.0225	0.0	0.7105	0.6763	0.9531
	21	25	25	20	21	21
L	0.84214	0.21760	-0.08855	1.00000	0.94551	0.10065
	0.0001	0.3186	0.7105	0.0	0.0001	0.6818
	21	23	20	23	23	19
B	0.82340	0.26154	-0.09682	0.94551	1.00000	0.09370
	0.0001	0.2170	0.6763	0.0001	0.0	0.6944
	22	24	21	23	24	20
ARHENIUS	0.19461	-0.03993	-0.01367	0.10065	0.09370	1.00000
	0.3979	0.8565	0.9531	0.6818	0.6944	0.0
	21	23	21	19	20	23

Table 5 (continued)

CONTROL SUBJECTS

The SAS System 1
17:21 Monday, August 15, 1994

General Linear Models Procedure

Number of observations in data set = 31

NOTE: Due to missing values, only 20 observations can be used in this analysis.

^L The SAS System 2
17:21 Monday, August 15, 1994

General Linear Models Procedure

Dependent Variable: INVSLOPE

Source	DF	Sum of Squares	F Value	Pr > F
Model	5	0.91110517	14.98	0.0001
Error	14	0.17028274		
Corrected Total	19	1.08138792		

R-Square	C.V.	INVSLOPE Mean
0.842533	23.23262	0.47470433

Source	DF	Type I SS	F Value	Pr > F
AGE	1	0.00138064	0.11	0.7412
HB	1	0.00000201	0.00	0.9899
L	1	0.87060687	71.58	0.0001
B	1	0.01989937	1.64	0.2217
ARHENIUS	1	0.01921628	1.58	0.2293

Source	DF	Type III SS	F Value	Pr > F
AGE	1	0.02128087	1.75	0.2071
HB	1	0.05661561	4.65	0.0488
L	1	0.12748733	10.48	0.0060
B	1	0.02115712	1.74	0.2084
ARHENIUS	1	0.01921628	1.58	0.2293

Parameter	Estimate	T for H0: Parameter=0	Pr > |T|	Std Error of Estimate
INTERCEPT	0.6823554672	2.06	0.0589	0.33187722
AGE	-.0046553958	-1.32	0.2071	0.00351952
HB	-.0245758863	-2.16	0.0488	0.01139101
L	0.1587893345	3.24	0.0060	0.04904662
B	-.0670789948	-1.32	0.2084	0.05086037

Table 5 (continued)

ARHENIUS	-.0000302064	-1.26	0.2293	0.00002403

^L The SAS System 3
17:21 Monday, August 15, 1994

Correlation Analysis

6 'VAR' Variables: INVSLOPE AGE HB L B ARHENIUS

Simple Statistics

Variable	N	Mean	Std Dev	Sum	Minimum	Maximum
INVSLOPE	28	0.4750	0.2214	13.3013	0.2414	1.1443
AGE	31	72.0694	8.3686	2234.2	60.7000	86.8300
HB	30	15.9585	2.1891	478.8	12.5175	21.9000
L	29	5.3364	2.4902	154.8	2.3864	11.2110
B	27	4.6250	2.2650	124.9	1.8420	9.8603
ARHENIUS	24	-451.5	1173.3	-10835.9	-3104.0	2005.8

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

	INVSLOPE	AGE	HB	L	B	ARHENIUS
INVSLOPE	1.00000	0.04319	0.04886	0.85303	0.80791	0.08947
	0.0	0.8273	0.8050	0.0001	0.0001	0.6921
	28	28	28	27	26	22
AGE	0.04319	1.00000	-0.30581	-0.04411	-0.11621	-0.44549
	0.8273	0.0	0.1003	0.8203	0.5638	0.0291
	28	31	30	29	27	24
HB	0.04886	-0.30581	1.00000	0.17768	0.17982	0.06658
	0.8050	0.1003	0.0	0.3565	0.3694	0.7573
	28	30	30	29	27	24
L	0.85303	-0.04411	0.17768	1.00000	0.97276	0.07299
	0.0001	0.8203	0.3565	0.0	0.0001	0.7407
	27	29	29	29	26	23
B	0.80791	-0.11621	0.17982	0.97276	1.00000	0.15884
	0.0001	0.5638	0.3694	0.0001	0.0	0.4802
	26	27	27	26	27	22
ARHENIUS	0.08947	-0.44549	0.06658	0.07299	0.15884	1.00000
	0.6921	0.0291	0.7573	0.7407	0.4802	0.0
	22	24	24	23	22	24

Table 5 (continued)

Table 6 Summary of correlations present between experimental parameters derived from blood of Alzheimer's patients

	1/slope (kcatEt/Km)*	Age (years)	Hb (g/dL)	Lowry (ug/mL)	Bradford (ug/mL)	Temp. Slope
1/slope (kcatEt/Km)*	*			*	*	
Age (years)		*				
Hb (g/dL)			*			
Lowry (ug/mL)	*			*	*	
Bradford (ug/mL)	*			*	*	
Temp. Slope						*

* denotes a statistically significant probability that a correlation exists between the two comparison groups ($p<0.01$)

Table 7 Summary of correlations present between experimental parameters derived from blood of control subjects

	1/slope (kcatEt/Km)	Age (years)	Hb (g/dL)	Lowry (ug/mL)	Bradford (ug/mL)	Temp. Slope
1/slope (kcatEt/Km)	*			*	*	
Age (years)		*				
Hb (g/dL)			*			
Lowry (ug/mL)	*			*	*	
Bradford (ug/mL)	*			*	*	
Temp. Slope						*

* denotes a statistically significant probability that a correlation exists between the two comparison groups ($p<0.01$)

OBS	AGE	HB	L	B	INVSLOPE	ARHENIU	S	FLAG
1	63.00	.	1.4153	1.07618	.	.		1
2	71.00	.	1.9996	1.63080	1.45631	-5854.4	3	1
3	80.00	.	2.2232	1.84741	1.78571	-413.5	8	1
4	75.57	13.9760	1.0987	0.90459	0.79787	.		1
5	79.00	16.1920	.	.	.	-9099.7	4	1
6	80.00	16.0880	0.8143	0.82688	0.58824	-1304.2	9	1
7	71.00	15.9360	0.6784	0.52493	0.66964	-401.6	0	1
8	66.00	14.6240	0.9185	0.90495	1.25900	-777.7	6	1
9	72.00	14.3040	.	.	.	.		1
10	77.00	14.2880	0.9054	0.86715	0.62500	-21487.7	7	1
11	76.00	17.1520	1.1432	1.10228	0.75758	.		1
12	76.00	18.1040	1.9818	1.56825	1.37615	-118.3	0	1
13	67.00	14.4960	0.9480	0.64845	0.96154	-280.2	1	1
14	67.00	15.2480	1.1738	1.09215	1.09489	-83.8	9	1
15	78.00	12.9920	.	.	.	-646.6	9	1
16	71.00	15.2880	1.5975	1.85198	1.50000	-673.0	0	1
17	84.00	11.2480	1.6709	1.53158	1.54639	672.3	1	1
18	68.00	16.6400	.	1.22243	1.30435	-1768.5	8	1
19	64.00	17.1760	0.5285	0.43695	0.36946	-97.3	5	1
20	84.00	14.2160	0.9421	0.77625	1.02041	-1083.3	3	1
21	97.00	11.0880	1.3579	1.26810	0.76923	-340.2	9	1
22	72.00	13.0960	1.3471	1.29555	0.96774	1145.9	9	1
23	76.00	18.2560	1.8070	1.62495	2.05479	-1257.8	8	1
24	81.00	11.2880	1.0150	0.92835	0.89286	-1402.2	6	1
25	60.00	17.4000	0.7238	0.57900	.	.		1
26	77.00	17.7760	1.1446	1.11038	0.81522	-1406.3		

Table 8 Output from SAS Program on analysis of variance between experimental parameters derived from blood of Alzheimer's patients and control subjects

4	1	27	69.00	18.9920	.	.	0.76142	-2257.9
3	1	28	82.00	18.1280	0.7553	0.74925	0.76531	-733.5
5	1	29	60.70	15.8175	3.8854	3.48300	0.44150	135.1
2	0	30	60.86	.	.	.	.	.
	0	31	60.86	21.9000	5.2476	4.39400	0.32091	845.3
8	0	32	63.00	16.2225	11.2110	9.56550	.	-784.9
7	0	33	63.44	12.5850	3.6462	3.54450	0.33317	-2299.2
7	0	34	63.78	17.7450	5.7366	5.31150	0.53637	-346.6
3	0	35	63.88	17.4600	3.2595	2.03400	0.50005	82.3
2	0	36	64.32	16.3425	4.0938	3.12750	0.27471	1190.9
6	0	37	65.15	18.2850	4.5258	4.44000	0.31986	347.8
9	0	38	66.00	16.3950	5.3808	4.52700	0.37010	367.2
9	0	39	67.00	17.2800	3.0298	.	0.30295	.
	0	40	67.00	15.1125	3.8900	3.58300	0.25135	.
	0	41	68.11	15.7125	5.5380	5.93505	0.52934	-609.0
4	0	42	68.16	14.9550	6.6078	5.58225	0.44423	.
	0	43	68.93	14.9400	3.8322	3.50100	0.53118	-802.0
8	0	44	69.79	18.3525	7.6780	.	0.81873	-780.2
9	0	45	70.60	13.2600	3.2691	1.84200	0.42391	335.3
8	0	46	71.38	17.2650	10.7631	9.86025	1.14425	-67.0
3	0	47	72.58	14.7225	.	5.15750	0.64289	-532.6
8	0	48	76.98	19.9350	7.5213	6.32125	0.58854	-598.0
7	0	49	78.04	15.2925	10.7748	9.80355	0.85135	550.4
3	0	50	79.00	13.2600	4.1748	4.42050	0.28769	2005.8
4	0	51	80.00	16.2675	4.2165	3.54600	0.39283	.
	0	52	80.00	14.5650	8.3845	7.00375	0.87925	-548.9
7	0	53	80.88	16.0725	3.8403	3.83400	0.27131	-1149.9
4	0	54	81.00	12.7500	3.2856	2.90250	0.37880	-668.9
0	0							

Table 8 (continued)

```
              55     82.00     14.7225     3.5571    4.16550     0.54085          .
         0
              56     84.00     12.5175     3.3300    2.06100     0.41334          .
         0
              57     84.00     16.5525     2.3864    2.07850     0.24143    -2253.2
4        0
              58     85.88     13.9500     7.8252       .           .       -2151.4
6        0
              59     86.83     18.5175     3.8652    2.85150     0.27037    -3104.0
2        0
^L                                               The SAS System         16:20 Monda
y, August 15, 1994   2

                                           Analysis of Variance Procedure
                                              Class Level Information

                                              Class     Levels    Values

                                              FLAG           2    0 1

                                       Number of observations in data set = 59

                                          Group    Obs    Dependent Variables

                                            1       59    AGE

                                            2       55    HB

                                            3       52    L

                                            4       51    B

                                            5       51    INVSLOPE

                                            6       47    ARHENIUS

NOTE: Variables in each group are consistent with respect to the presence or abs
ence of missing
      values.
                                                 The SAS System         16:20 Monday,
 August 15, 1994   3

                                           Analysis of Variance Procedure

Dependent Variable: AGE

Source                       DF             Sum of Squares             Mean Square
  F Value      Pr > F

Model                         1                80.82223270             80.82223270
     1.23      0.2729

Error                        57              3758.69259781             65.94197540
```

Table 8 (continued)

```
Corrected Total          58                 3839.51483051

                R-Square                       C.V.                  Root MSE
            AGE Mean

                0.021050                   11.09631                8.12046645
          73.18169492


Source                    DF                Anova SS              Mean Square
 F Value      Pr > F

FLAG                       1               80.82223270             80.82223270
     1.23      0.2729
```



```
                              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  df= 57  MSE= 65.94198
                                 Critical Value of T= 2.00
                              Least Significant Difference= 4.2395
                               WARNING: Cell sizes are not equal.
                              Harmonic Mean of cell sizes= 29.42373

               Means with the same letter are not significantly different.

                     T Grouping              Mean       N  FLAG

                              A            74.413      28  1
                              A
                              A            72.069      31  0
```



```
                              Analysis of Variance Procedure

Dependent Variable: HB

Source                    DF            Sum of Squares            Mean Square
 F Value      Pr > F

Model                      1               4.88980081              4.88980081
    0.98       0.3269
```

Table 8 (continued)

```
Error                       53            264.70629144          4.99445833

Corrected Total             54            269.59609225

                      R-Square                   C.V.              Root MSE
                 HB Mean

                      0.018138               14.24700            2.23482848
            15.68630909


Source                      DF                Anova SS           Mean Square
  F Value      Pr > F

FLAG                         1              4.88980081            4.88980081
      0.98      0.3269
```

```
                                          The SAS System          16:20 Monday,
 August 15, 1994   6

                                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= 53  MSE= 4.994458
                                      Critical Value of T= 2.01
                                 Least Significant Difference= 1.2139
                                  WARNING: Cell sizes are not equal.
                                Harmonic Mean of cell sizes= 27.27273

                        Means with the same letter are not significantly different.

                               T Grouping            Mean       N  FLAG

                                        A         15.9585      30  0
                                        A
                                        A         15.3597      25  1
```

```
                                          The SAS System          16:20 Monday,
 August 15, 1994   7

                                Analysis of Variance Procedure

Dependent Variable: L

Source                      DF          Sum of Squares           Mean Square
  F Value      Pr > F
```

Table 8 (continued)

```
Model                         1              216.75656880               216.75656880
    60.75       0.0001

Error                        50              178.40394452                 3.56807889

Corrected Total              51              395.16051332

                       R-Square                      C.V.                   Root MSE
                  L Mean

                       0.548528                  53.69051                 1.88893591
              3.51819351


Source                       DF                  Anova SS                Mean Square
  F Value       Pr > F

FLAG                          1              216.75656880               216.75656880
    60.75       0.0001


                                              The SAS System           16:20 Monday,
 August 15, 1994    8

                                     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= 50  MSE= 3.568079
                                      Critical Value of T= 2.01
                                 Least Significant Difference= 1.0594
                                  WARNING: Cell sizes are not equal.
                                Harmonic Mean of cell sizes= 25.65385

                      Means with the same letter are not significantly different.

                              T Grouping              Mean       N  FLAG

                                       A            5.3364      29  0

                                       B            1.2256      23  1


                                              The SAS System           16:20 Monday,
 August 15, 1994    9

                                     Analysis of Variance Procedure

Dependent Variable: B

Source                       DF            Sum of Squares                Mean Square
```

Table 8 (continued)

```
  F Value      Pr > F

Model                     1              157.99884710          157.99884710
     56.40       0.0001

Error                    49              137.26681680            2.80136361

Corrected Total          50              295.26566390

                   R-Square                      C.V.              Root MSE
             B Mean

                   0.535107                  56.43835            1.67372746
          2.96558525


Source                   DF                  Anova SS           Mean Square
  F Value      Pr > F

FLAG                      1              157.99884710          157.99884710
     56.40       0.0001


                                      The SAS System          16:20 Monday,
 August 15, 1994  10

                           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  df= 49  MSE= 2.801364
                           Critical Value of T= 2.01
                       Least Significant Difference= 0.9436
                        WARNING: Cell sizes are not equal.
                      Harmonic Mean of cell sizes= 25.41176

                   Means with the same letter are not significantly different.

                         T Grouping           Mean      N  FLAG

                                  A         4.6250     27  0

                                  B         1.0987     24  1


                                      The SAS System          16:20 Monday,
 August 15, 1994  11

                           Analysis of Variance Procedure

Dependent Variable: INVSLOPE
```

Table 8 (continued)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	4.16741517	4.16741517	39.32	0.0001
Error	49	5.19400832	0.10600017		
Corrected Total	50	9.36142349			

R-Square	C.V.	Root MSE	INVSLOPE Mean
0.445169	44.34895	0.32557667	0.73412494

Source	DF	Anova SS	Mean Square	F Value	Pr > F
FLAG	1	4.16741517	4.16741517	39.32	0.0001

Analysis of Variance Procedure

T tests (LSD) for variable: INVSLOPE

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

Alpha= 0.05 df= 49 MSE= 0.106
Critical Value of T= 2.01
Least Significant Difference= 0.1841
WARNING: Cell sizes are not equal.
Harmonic Mean of cell sizes= 25.2549

Means with the same letter are not significantly different.

T Grouping	Mean	N	FLAG
A	1.04953	23	1
B	0.47505	28	0

Analysis of Variance Procedure

Table 8 (continued)

Dependent Variable: ARHENIUS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	34265980.13724690	34265980.13724690	2.94	0.0932
Error	45	524164670.82958500	11648103.79621300		
Corrected Total	46	558430650.96683200			

R-Square	C.V.	Root MSE	ARHENIUS Mean
0.061361	-265.1088	3412.93184758	-1287.37004258

Source	DF	Anova SS	Mean Square	F Value	Pr > F
FLAG	1	34265980.13724700	34265980.13724700	2.94	0.0932

Analysis of Variance Procedure

T tests (LSD) for variable: ARHENIUS

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

Alpha= 0.05 df= 45 MSE= 11648104
Critical Value of T= 2.01
Least Significant Difference= 2005.8
WARNING: Cell sizes are not equal.
Harmonic Mean of cell sizes= 23.48936

Means with the same letter are not significantly different.

T Grouping	Mean	N	FLAG
A	-451.5	24	0
A			
A	-2159.6	23	1

Table 8 (continued)

Table 9 Variance between experimental parameters derived from blood of Alzheimer's patients and control subjects

	Alzheimer's Patients vs. Control Subjects
1/slope (kcatEt/Km)'	*
Age (years)	
Hb (g/dL)	
Lowry (ug/mL)	*
Bradford (ug/mL)	*
Temp. Slope	**

* denotes a statistically significant difference exists within a 95% confidence interval ($p<0.05$)

** denotes a statistically significant difference exists within a 90% confidence interval ($p<0.1$)

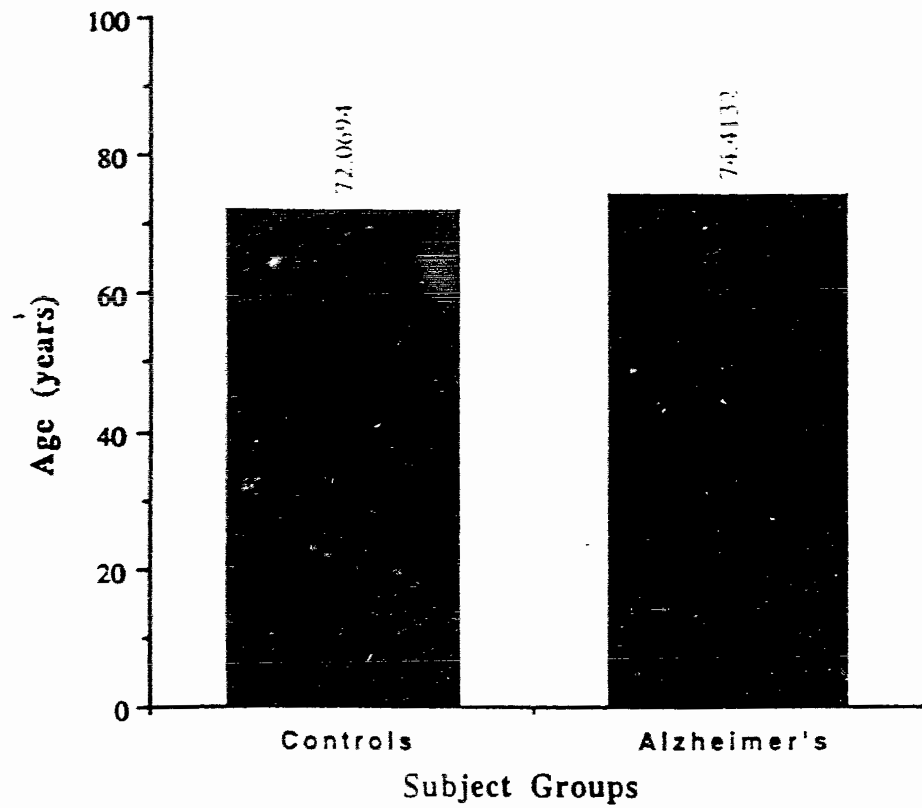
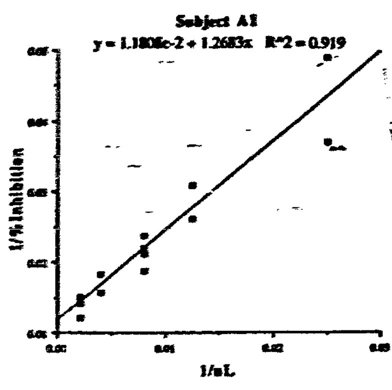
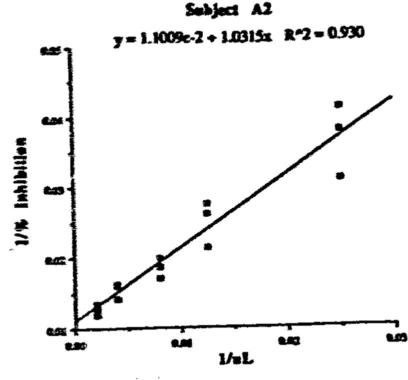
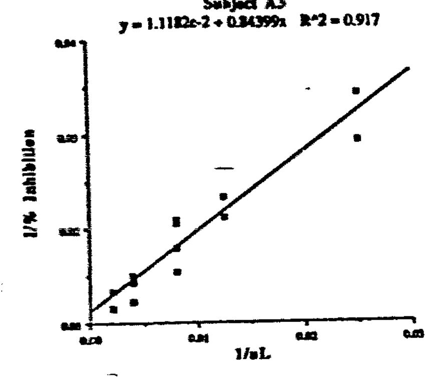
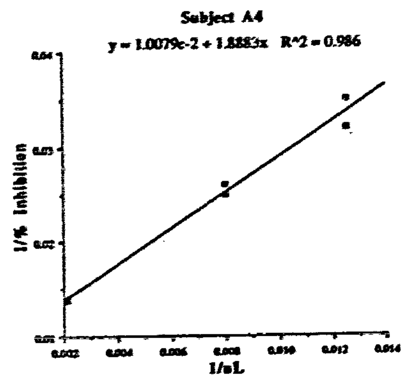


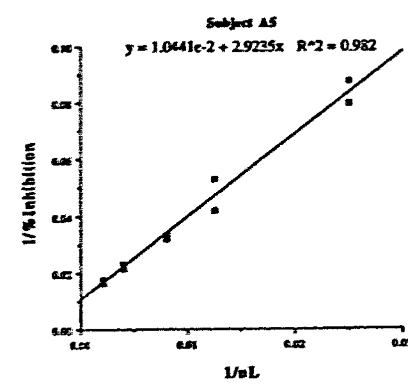
Figure 1. Comparison of age (in years) of Alzheimer's Patients and Control Subjects

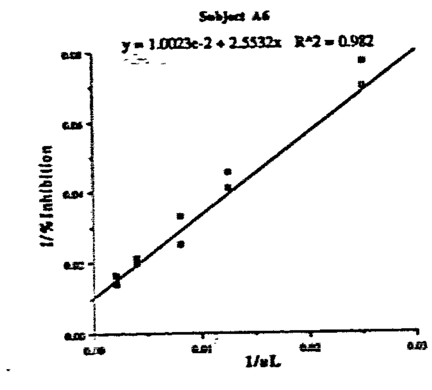


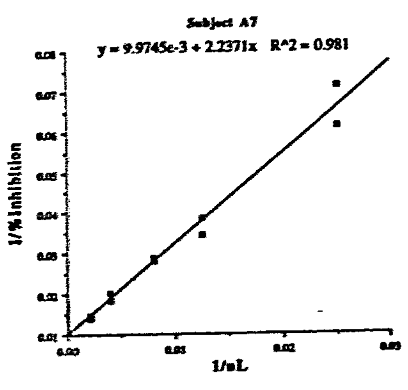


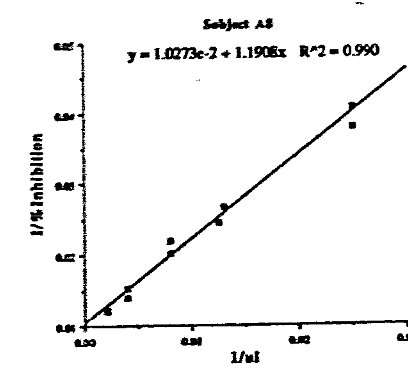


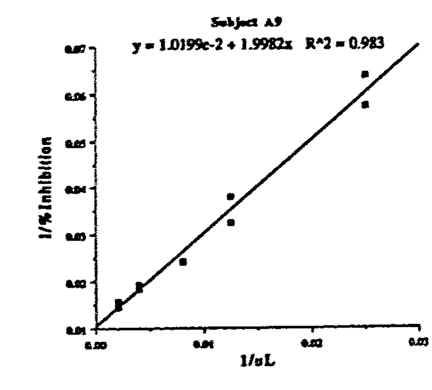


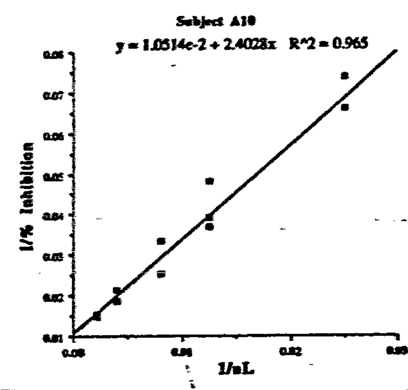


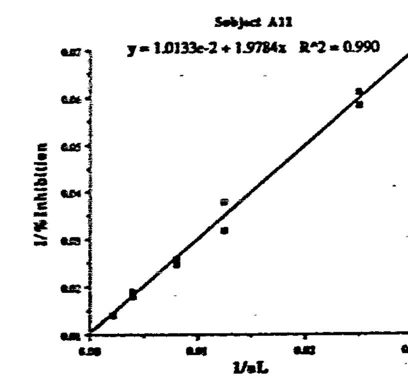












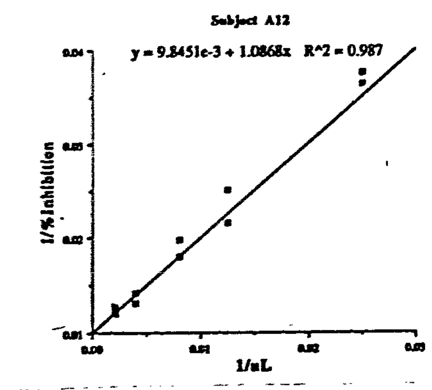
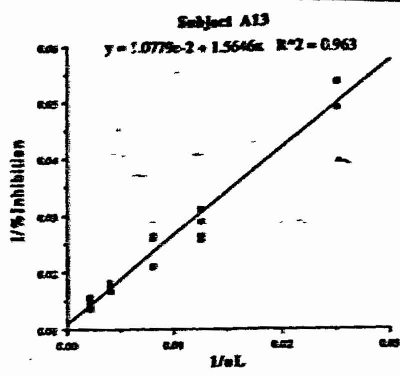
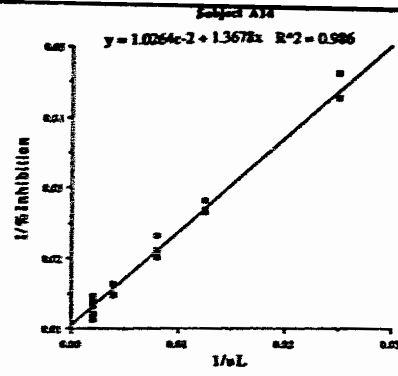
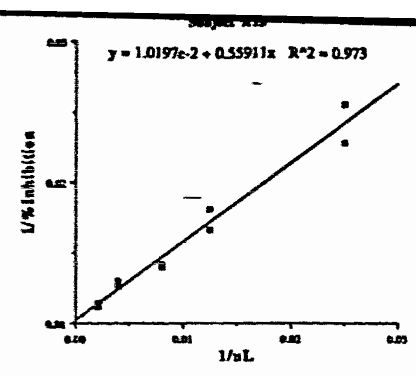
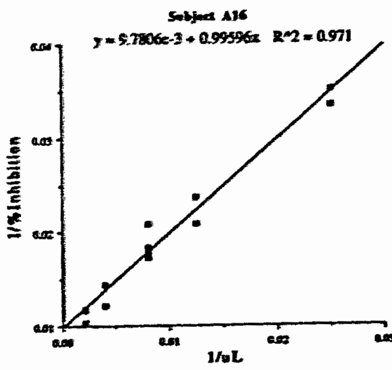


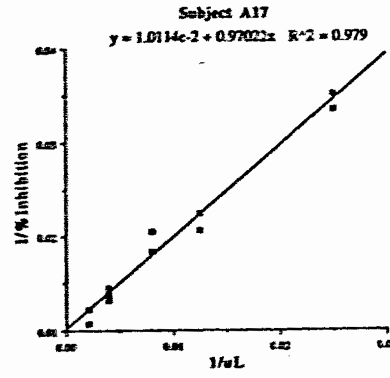
Figure 2. Summary of 1/% Inhibition vs. 1/uL SOD Graphs for Alzheimer's Patients

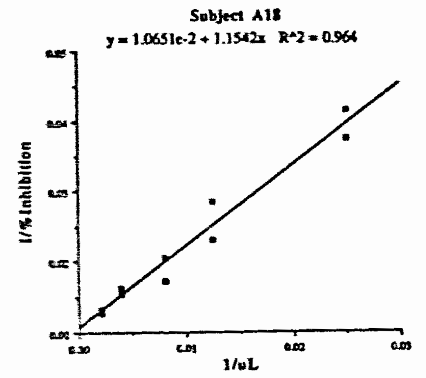


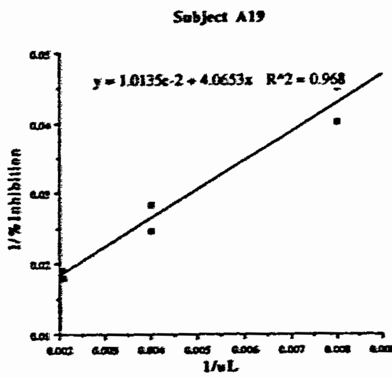


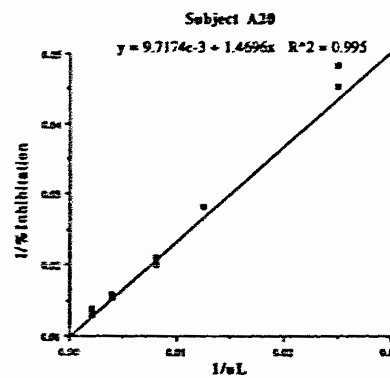


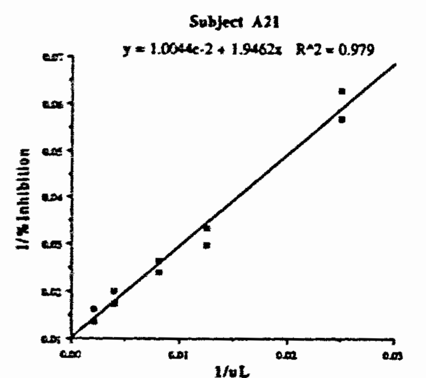


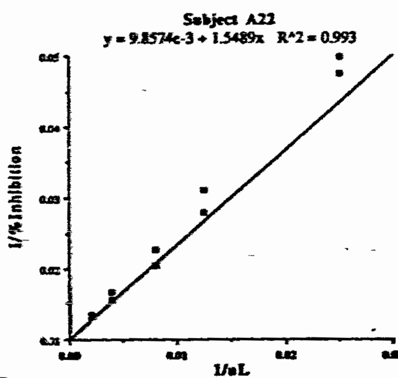


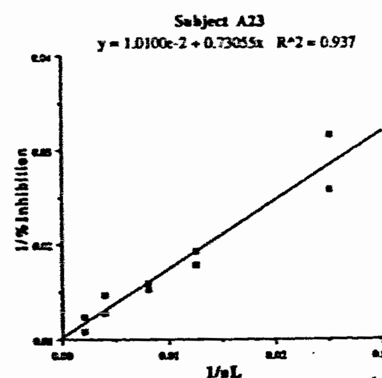












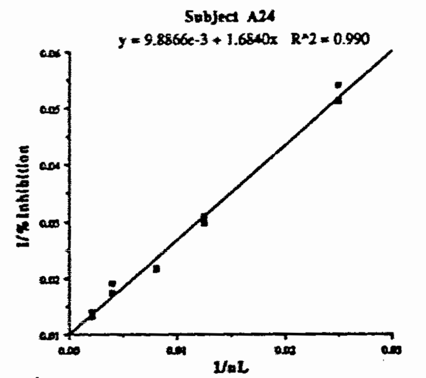
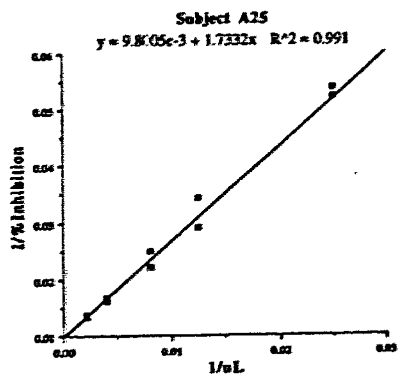
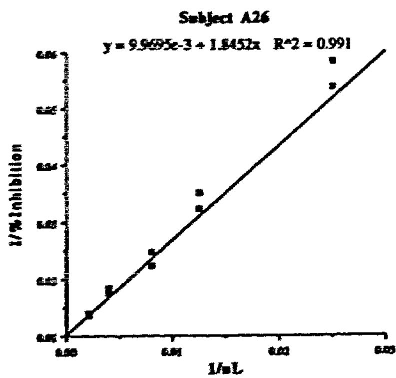
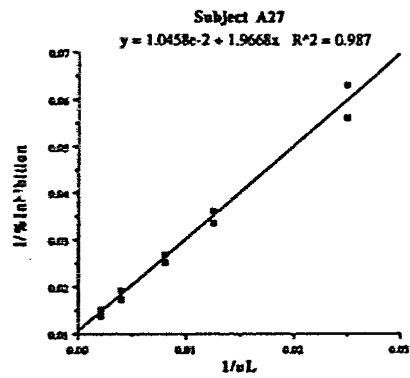
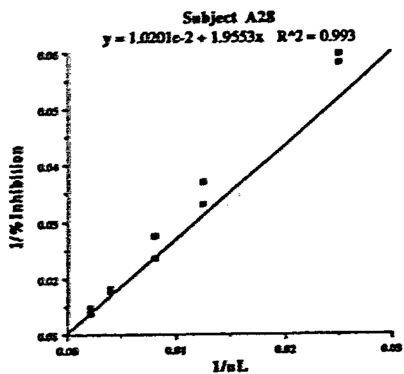


Figure 2. (continued)









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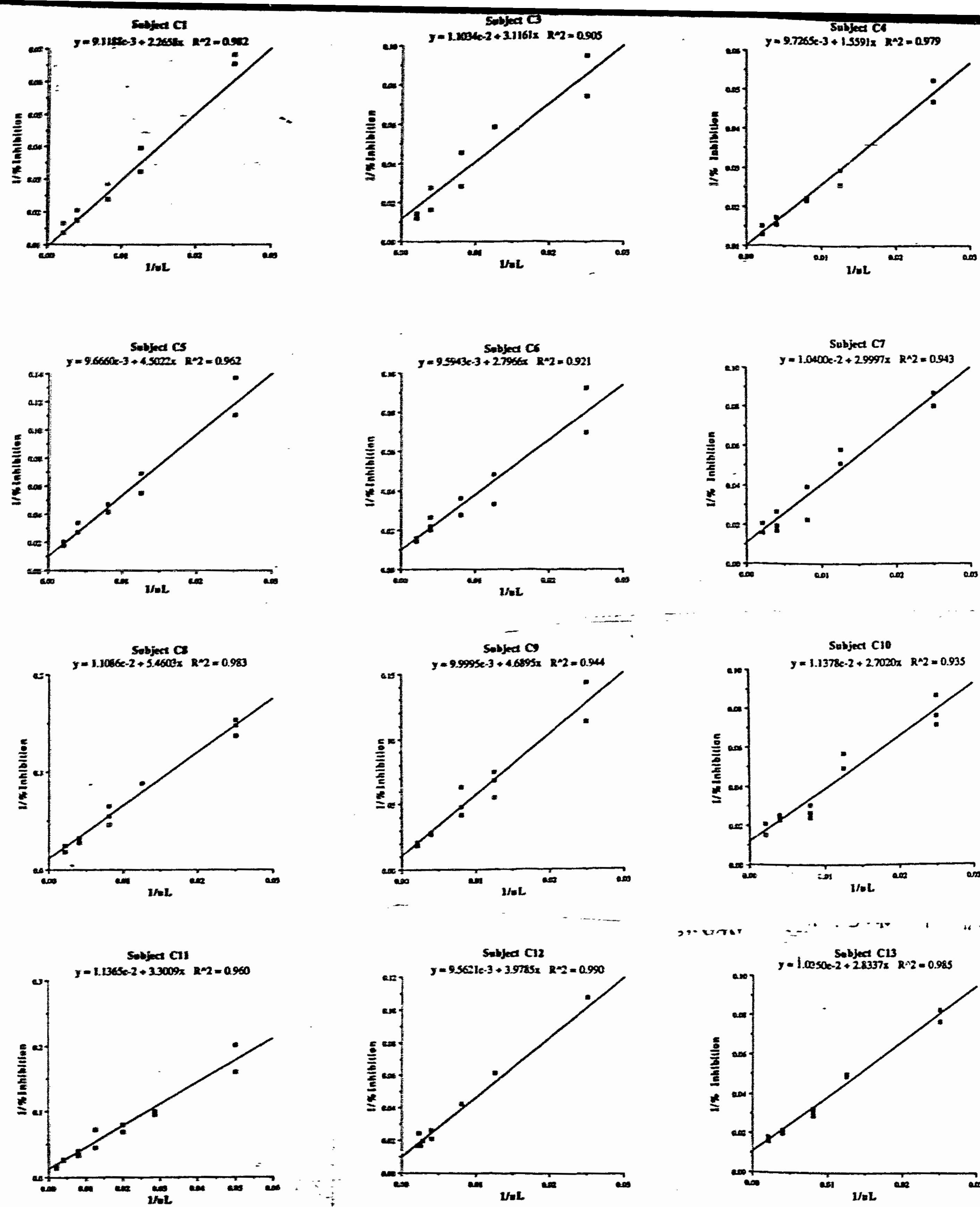
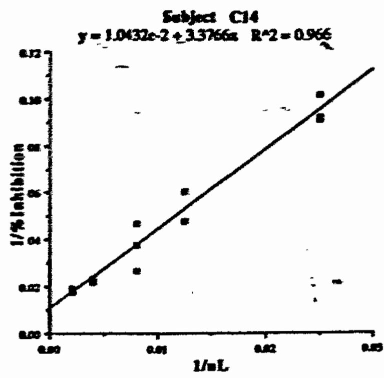
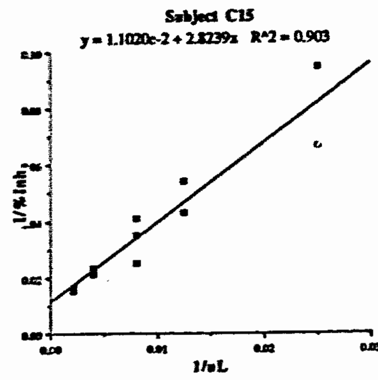
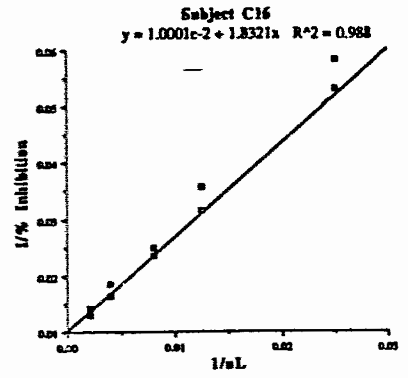
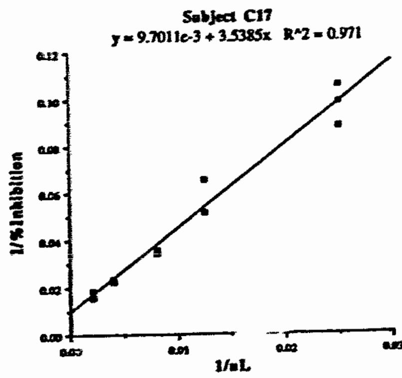


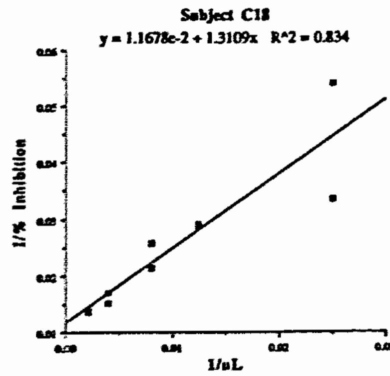
Figure 3. Summary of 1/% Inhibition vs. 1/uL SOD Graphs for Control Subjects

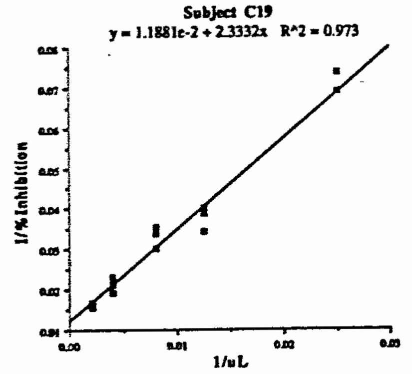


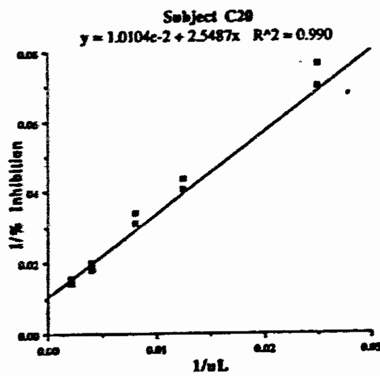


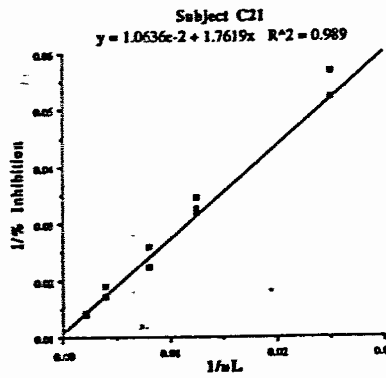


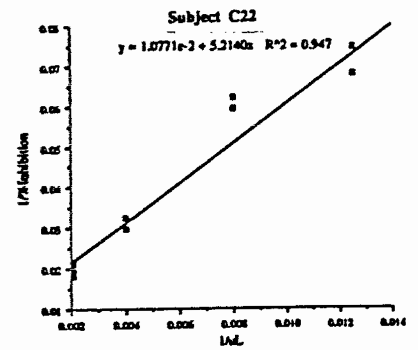


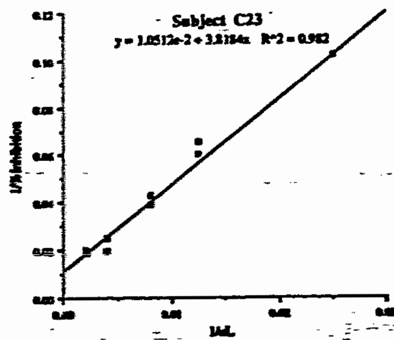


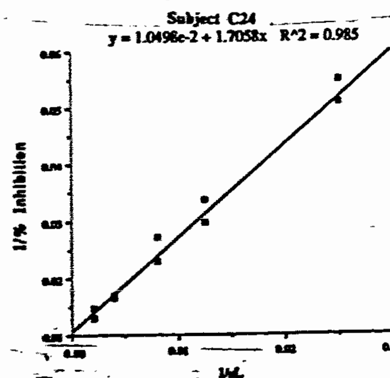


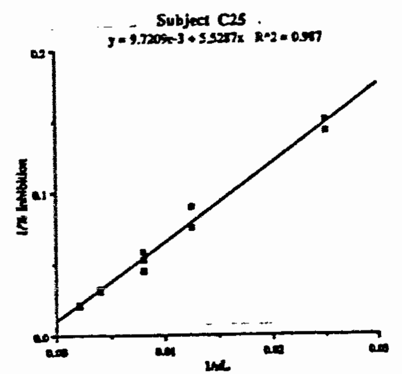












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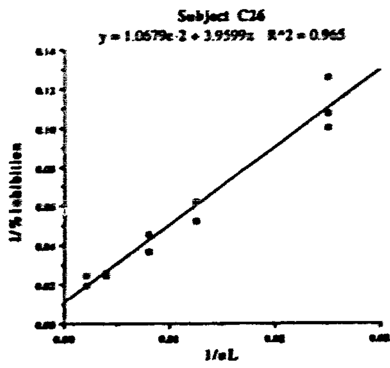
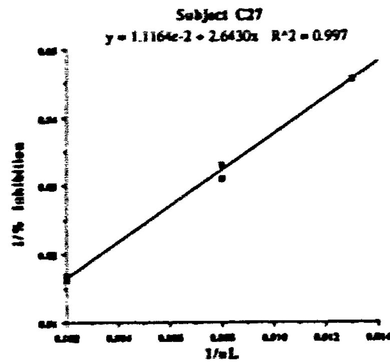
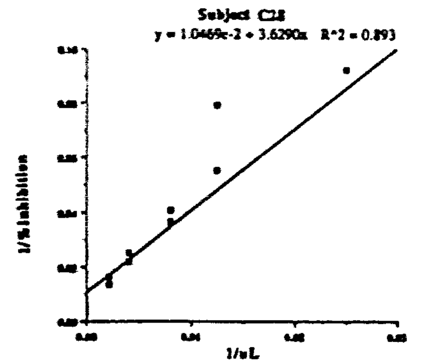
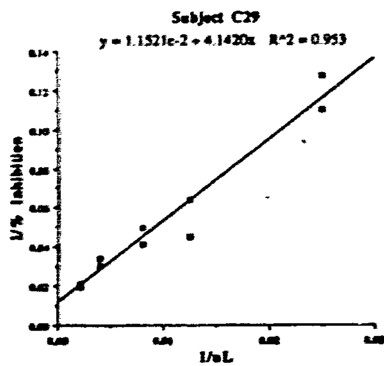
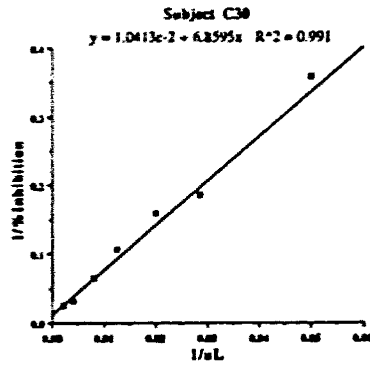










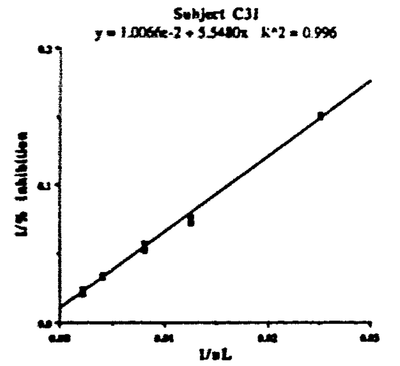


Figure 3. (continued)

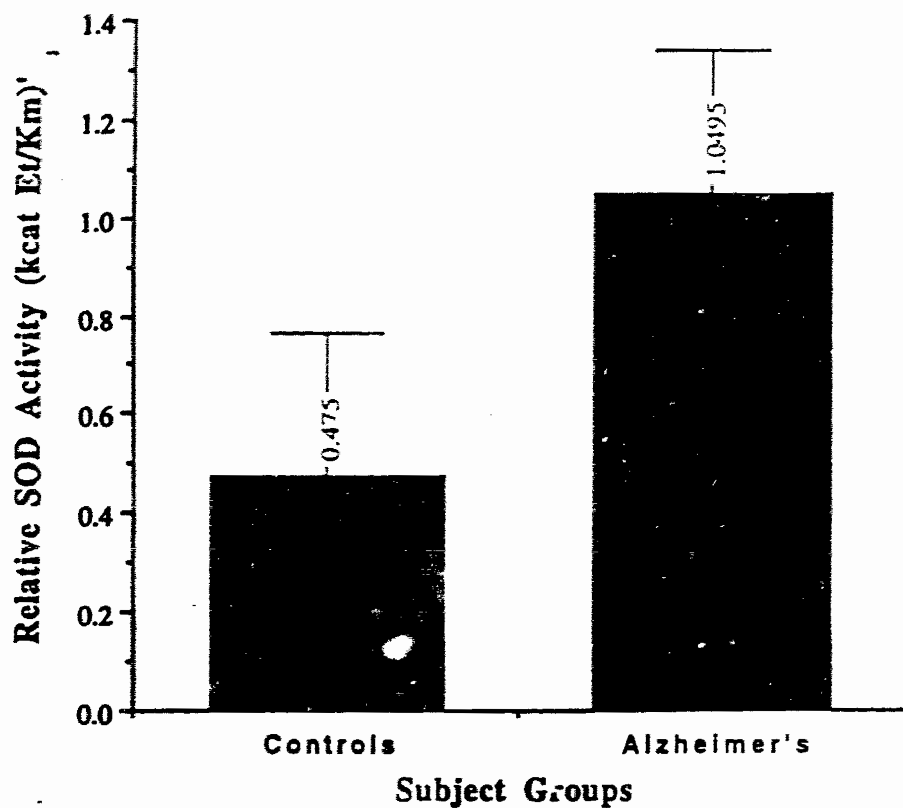


Figure 4. Relative Superoxide Dismutase Activities in Erythrocyte Extracts From Alzheimer's Patients and Control Subjects

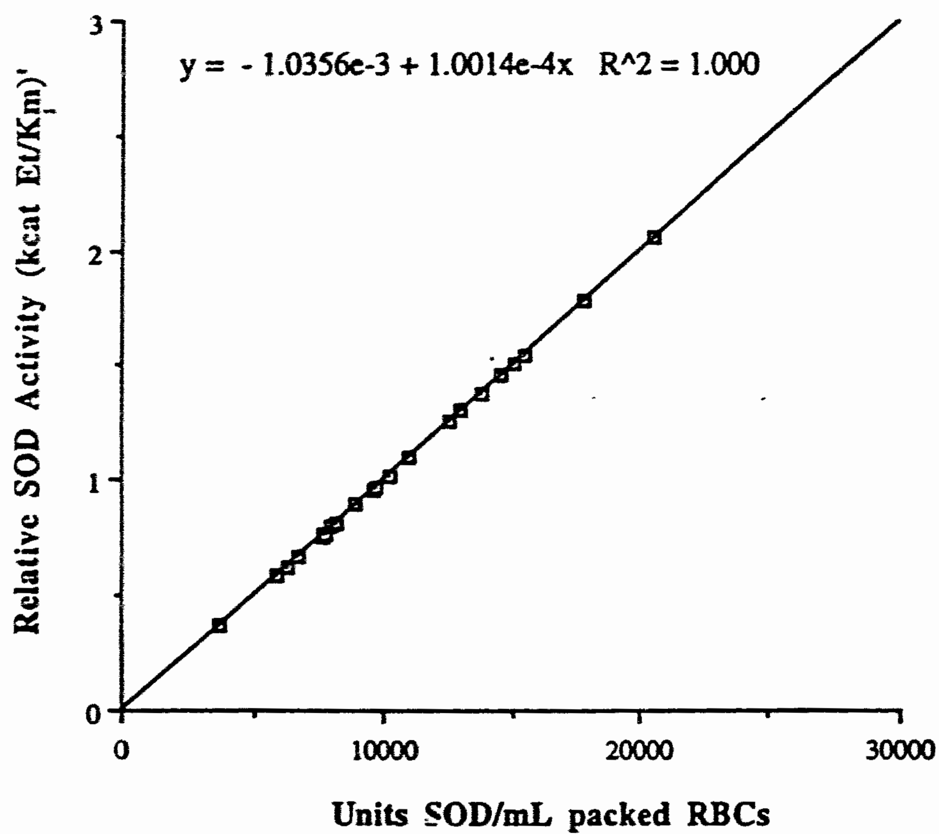


Figure 5. Relation of Relative SOD Activity to Total Activity in Erythrocytes of Alzheimer's Patients

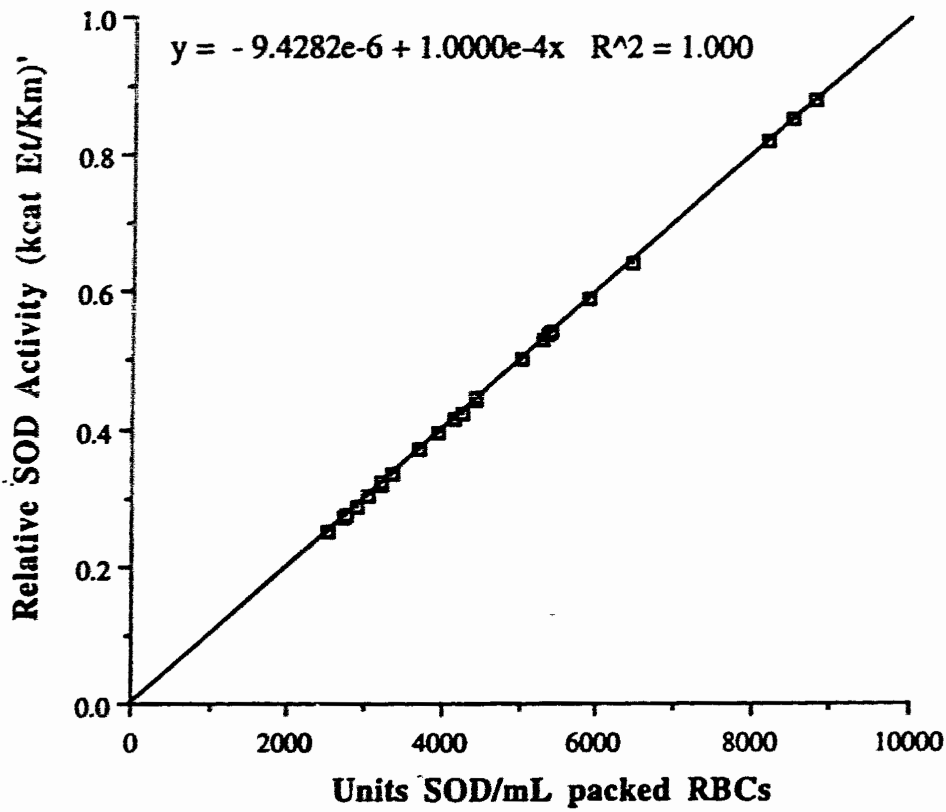


Figure 6. Relation of Relative SOD Activity to Total Activity in Erythrocytes of Control Subjects

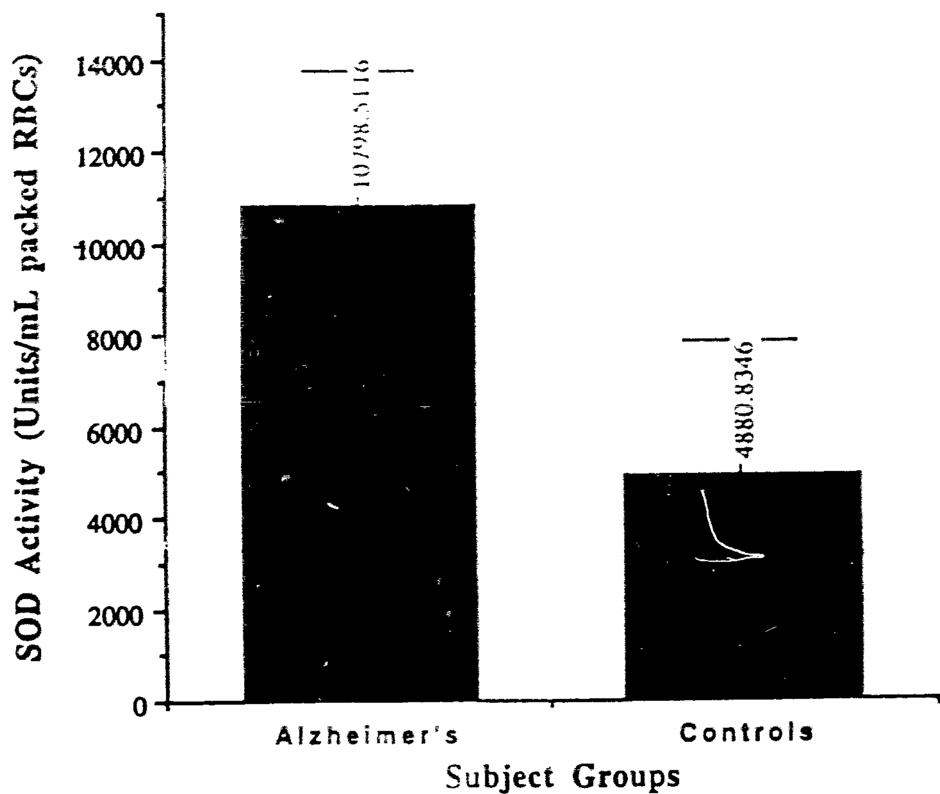


Figure 7. Superoxide Dismutase Activities in Erythrocytes From Alzheimer's Patients and Control Subjects

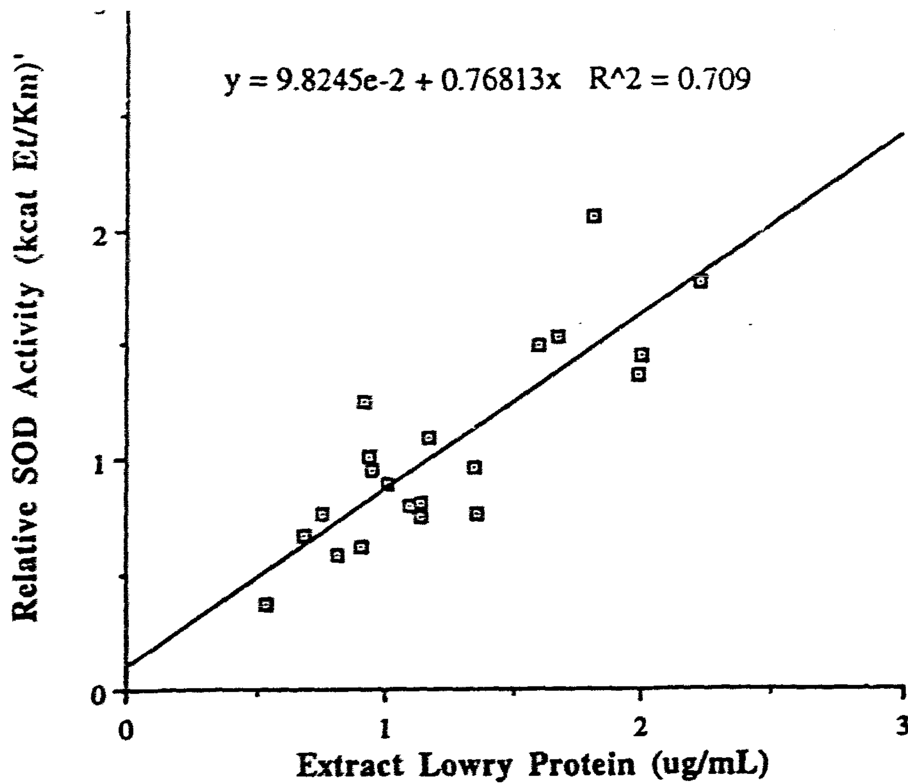


Figure 8A Erythrocyte Relative SOD Activity in Alzheimer's Patients Related to Total Protein Surviving the Chloroform/Ethanol Extraction

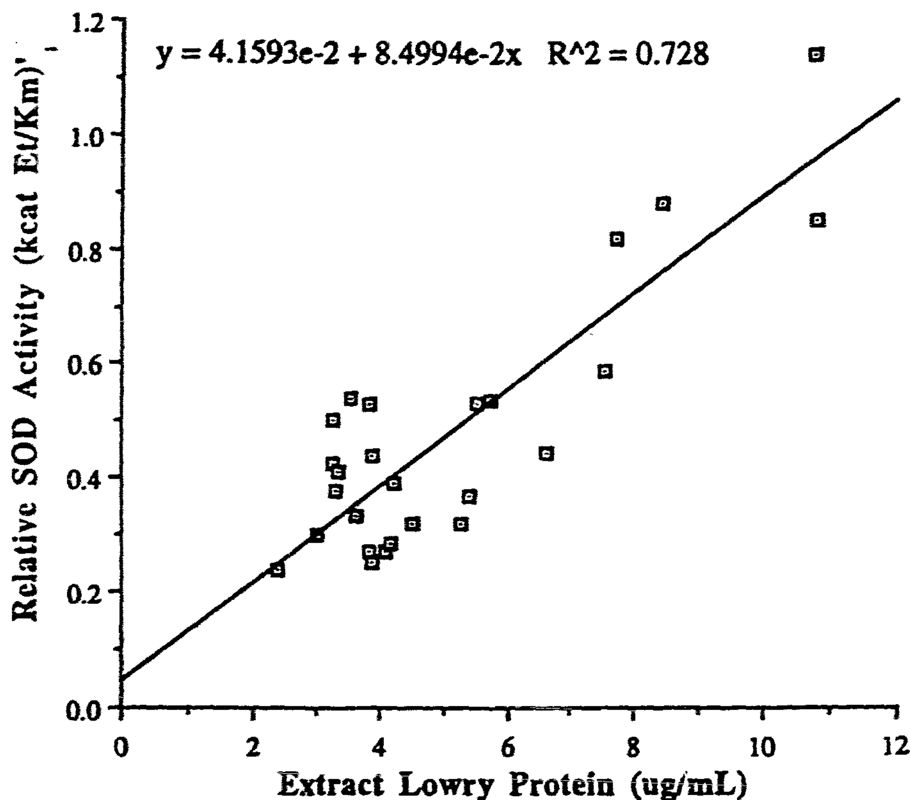


Figure 8B Erythrocyte Relative SOD Activity in Control Subjects Related to Total Protein Surviving the Chloroform/Ethanol Extraction

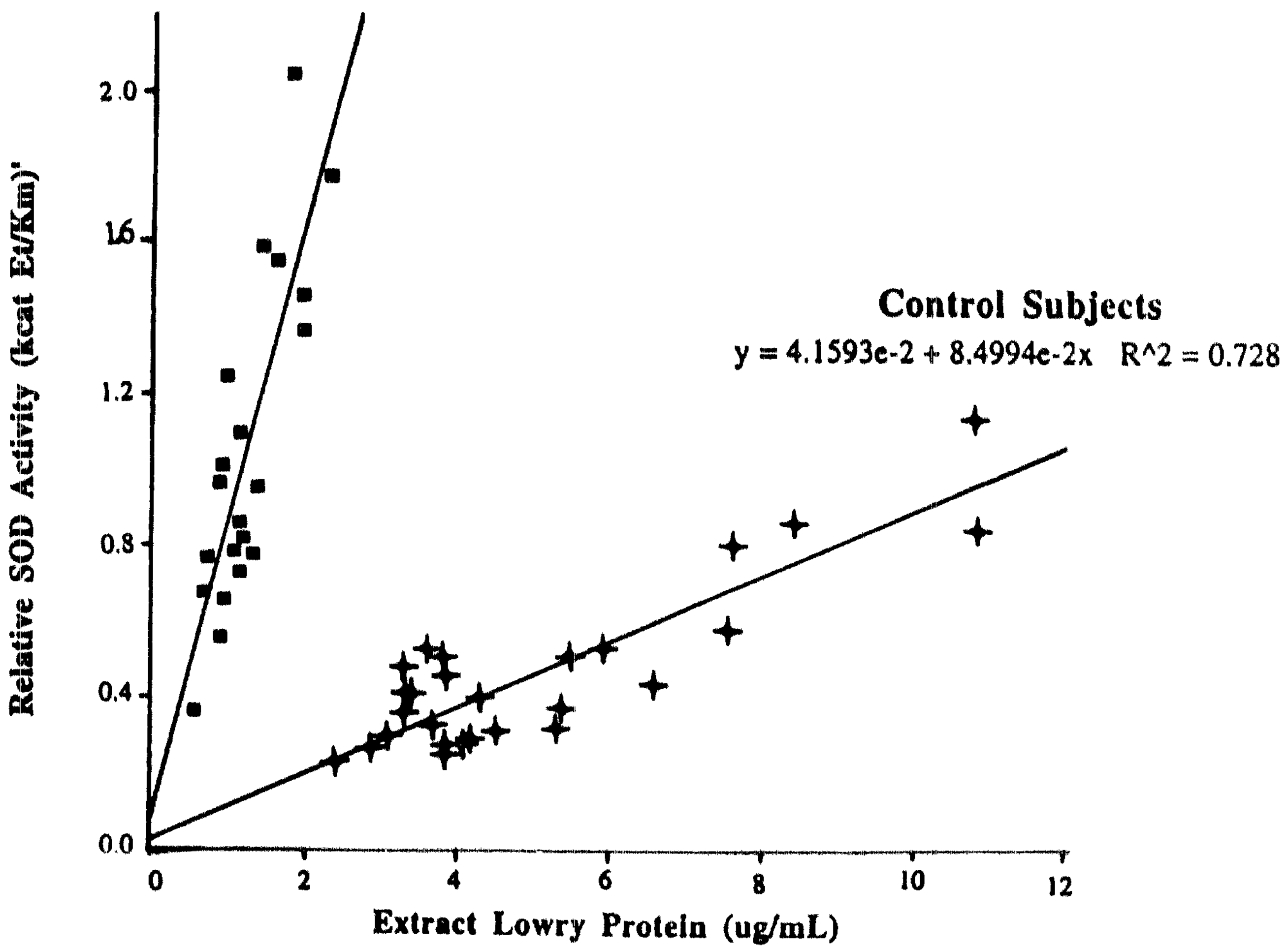


Figure 9. Relation of Relative SOD Activity (kcat Et/Km)' per Extract Lowry Protein in Alzheimer's Patients and Control Subjects

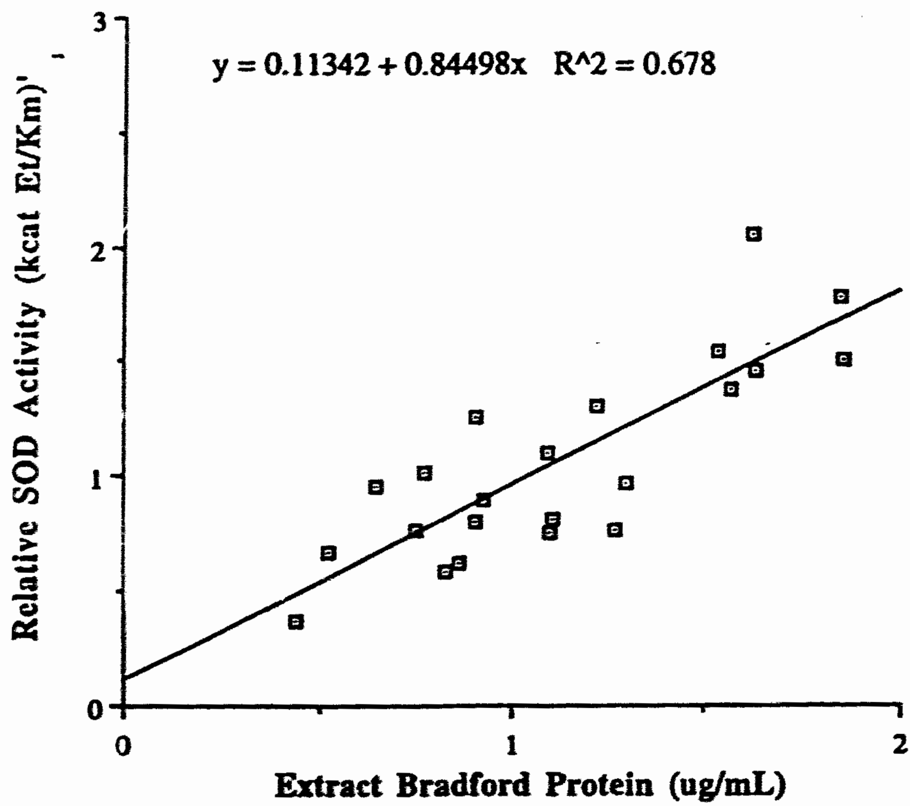


Figure 10. Erythrocyte Relative SOD Activity in Alzheimer's Patients Related to Total Protein Surviving the Chloroform/Ethanol Extraction

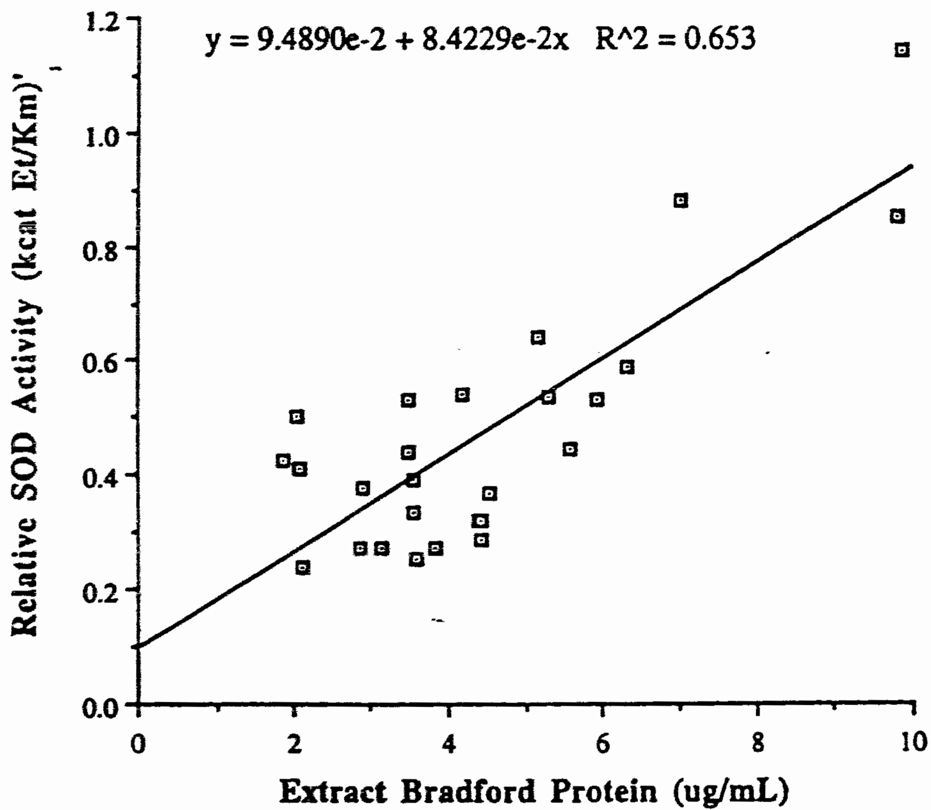


Figure 11. Erythrocyte Relative SOD Activity in Control Subjects Related to Total Protein Surviving the Chloroform/Ethanol Extraction

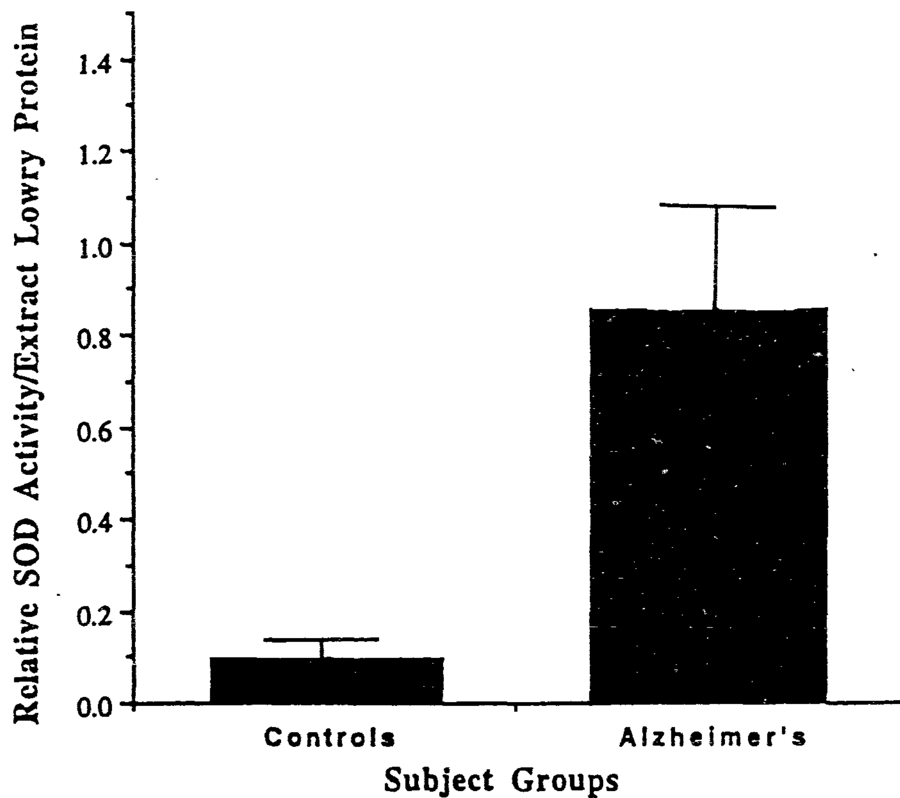


Figure 12. Relation of Relative SOD Activity (kcat Et/Km)' per Extract Lowry Protein in Alzheimer's Patients and Control Subjects

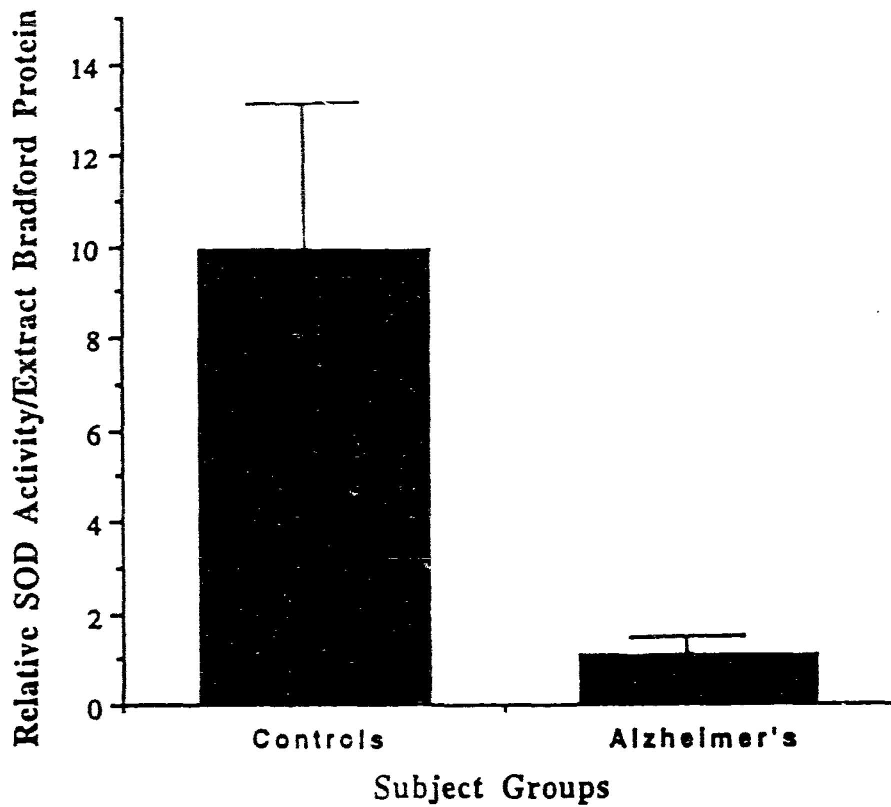


Figure 13. Relation of Relative SOD Activity (kcat Et/Km)' per Extract Bradford Protein in Alzheimer's Patients and Control Subjects

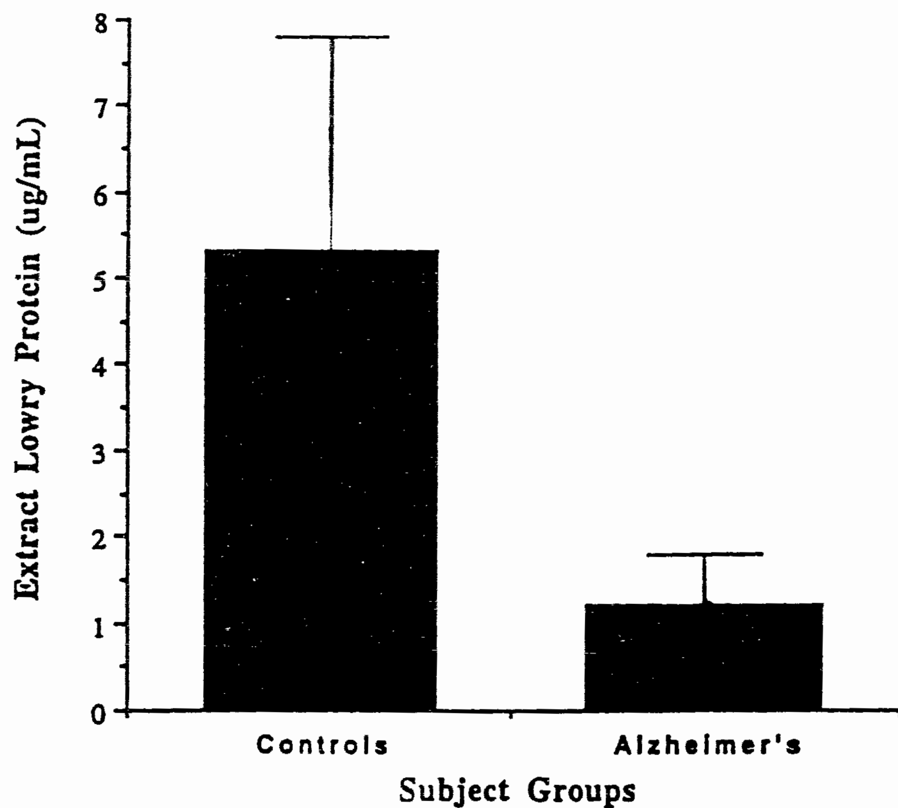


Figure 14. Relative Amounts of Lowry Protein Surviving Chloroform/Ethanol Extraction of Erythrocytes From Alzheimer's Patients and Controls

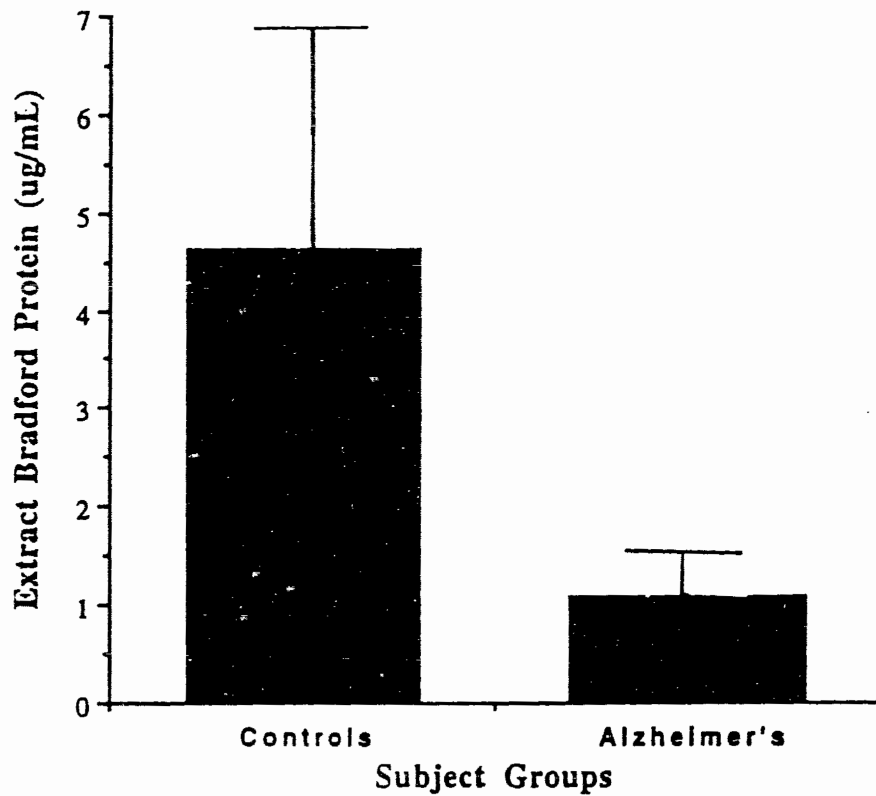


Figure 15. Relative Amounts of Bradford Protein Surviving Chloroform/Ethanol Extraction of Erythrocytes From Alzheimer's Patients and Controls

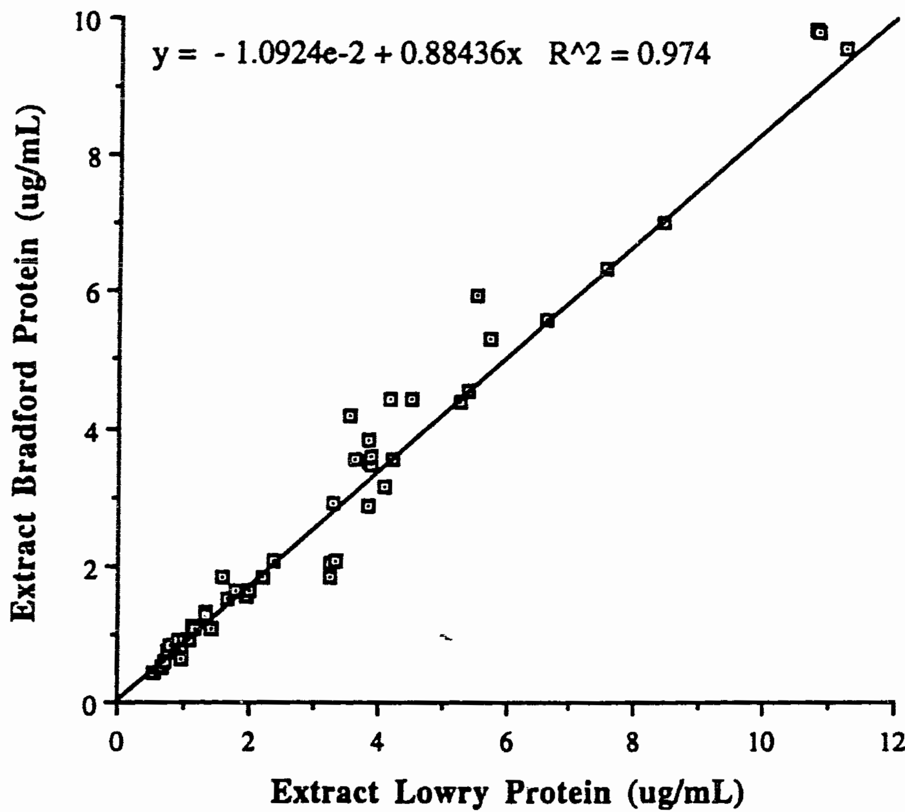


Figure 16. Relation Between Estimates of Protein Determined by Bradford and Lowry Methods in RBC Extracts

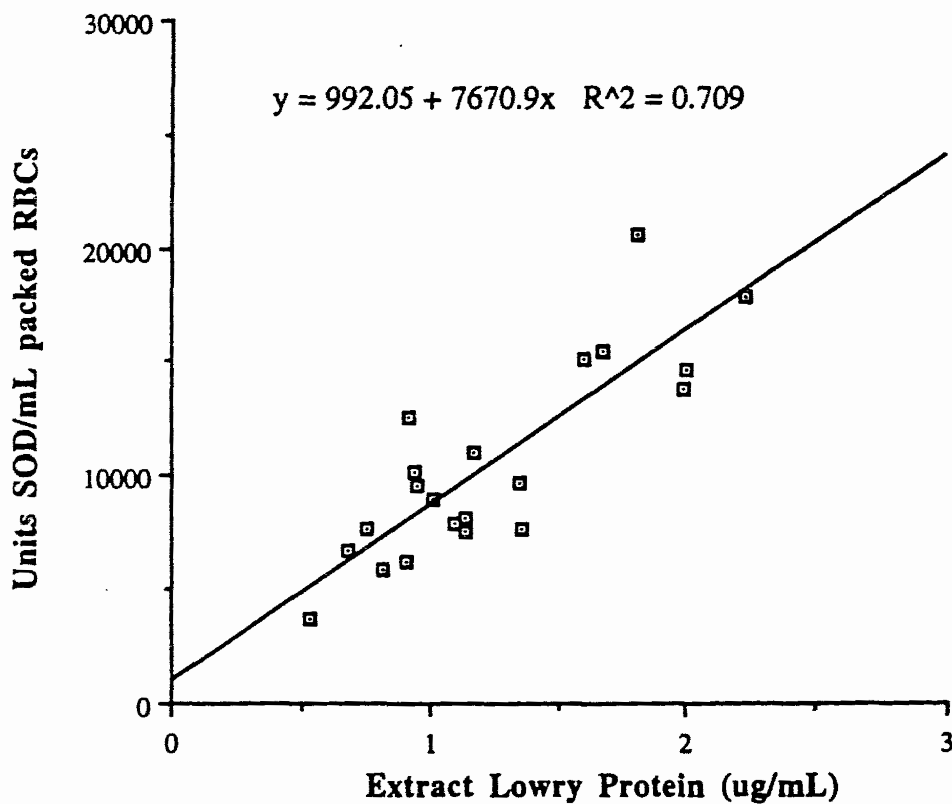


Figure 17. Erythrocyte SOD Activity Related to Total Protein Surviving Chloroform/Ethanol Extraction in Alzheimer's Patients

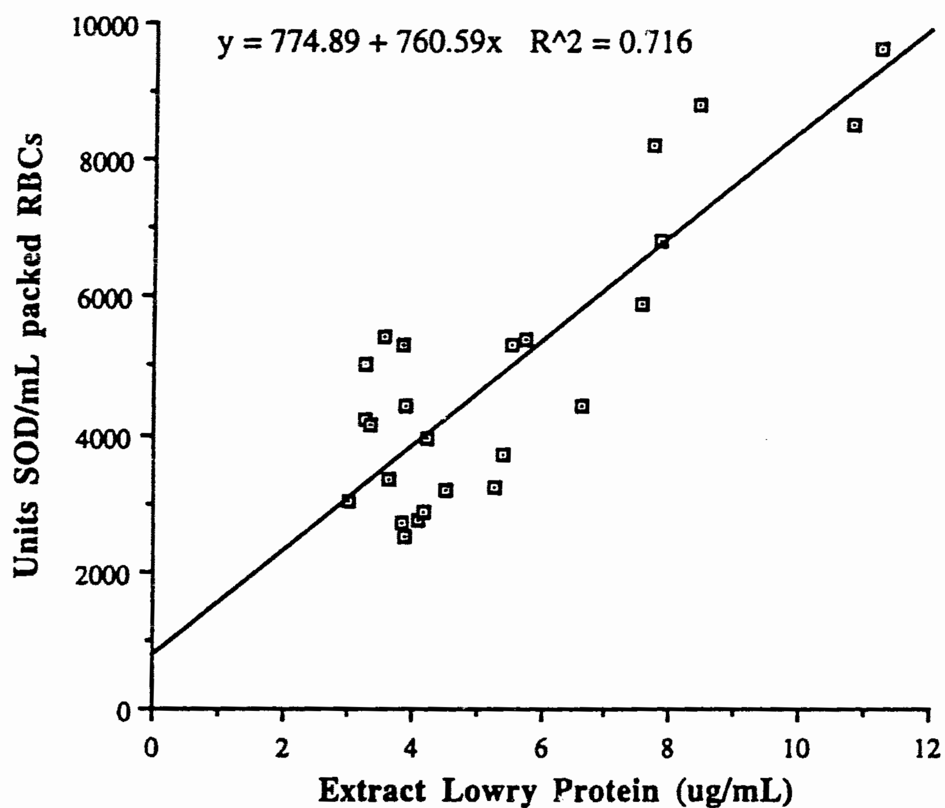


Figure 18. Erythrocyte SOD Activity Related to Total Protein Surviving Chloroform/Ethanol Extraction in Control Subjects

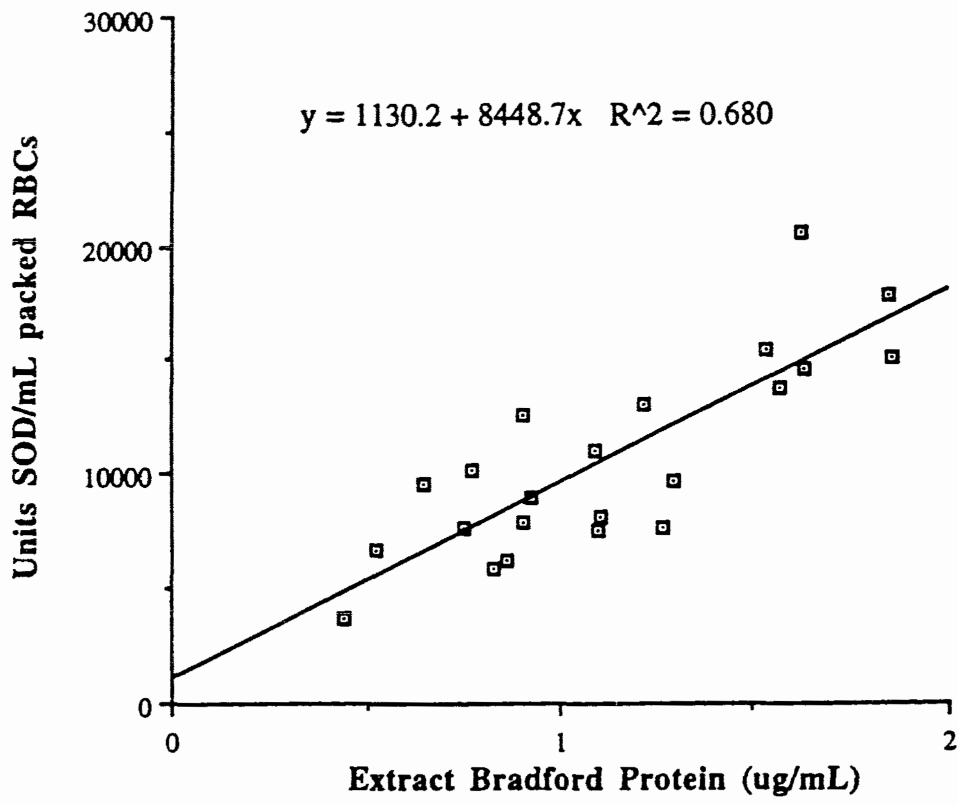


Figure 19. Erythrocyte SOD Activity Related to Total Protein Surviving Chloroform/Ethanol Extraction in Alzheimer's Patients

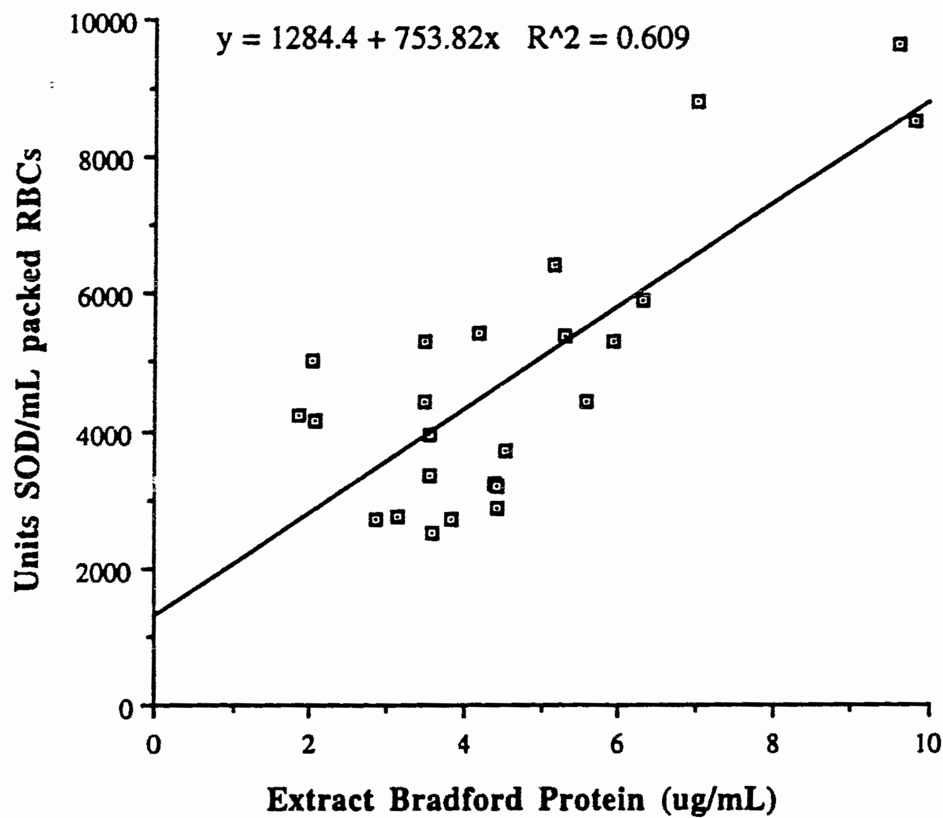
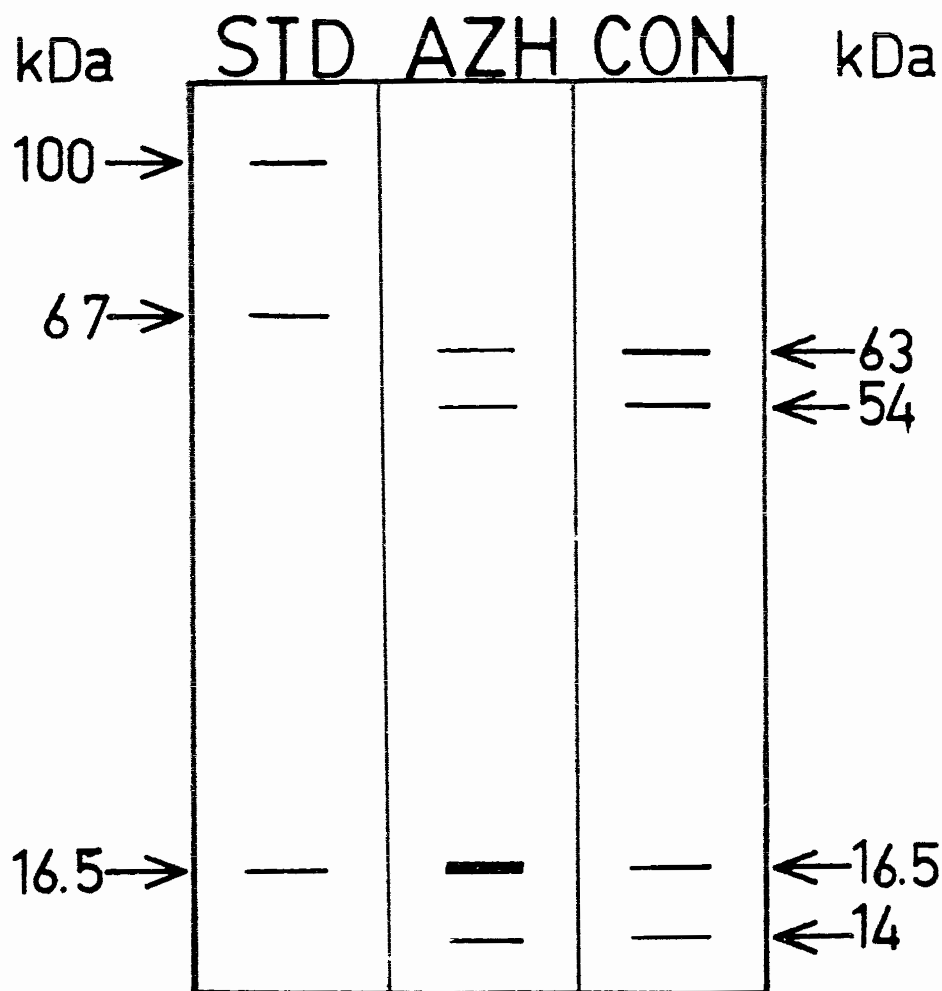


Figure 20. Erythrocyte SOD Activity Related to Total Protein Surviving Chloroform/Ethanol Extraction in Control Subjects



GEL ELECTROPHORESIS OF
EXTRACT PROTEINS

Figure 21. SDS-Gel Electrophoresis of Proteins in Chloroform/Ethanol Extract

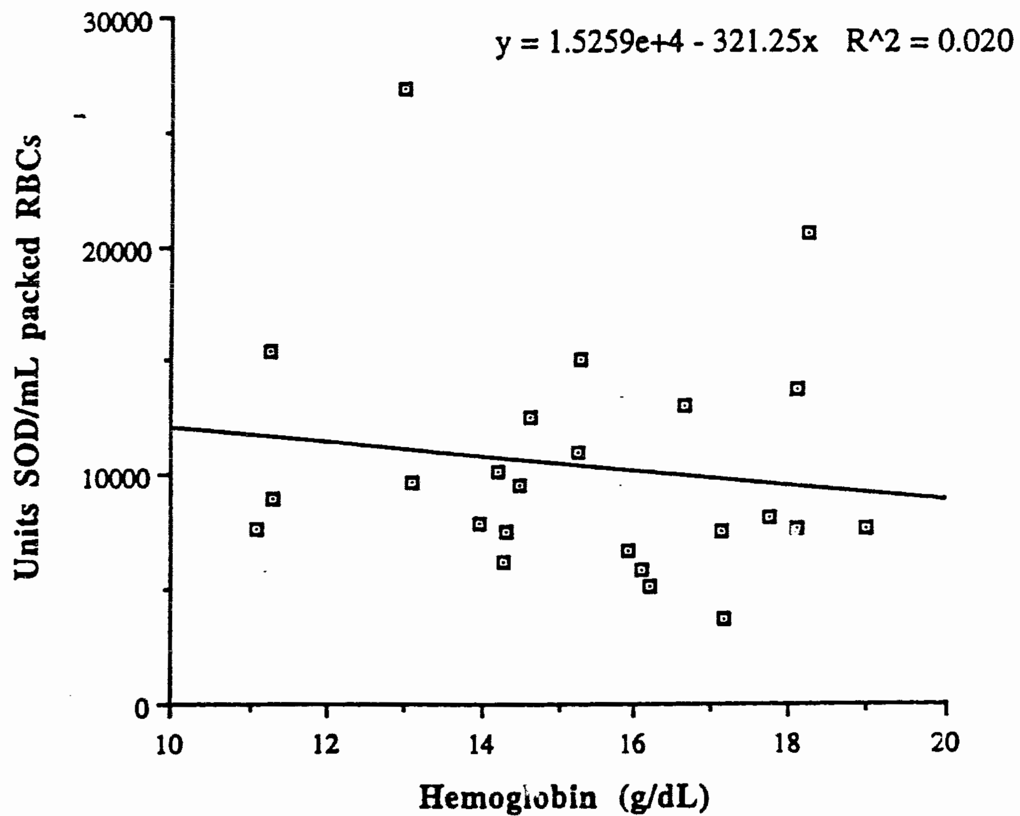


Figure 22. SOD Activity as a Function of Hemoglobin Content in Alzheimer's Patient's Erythrocytes

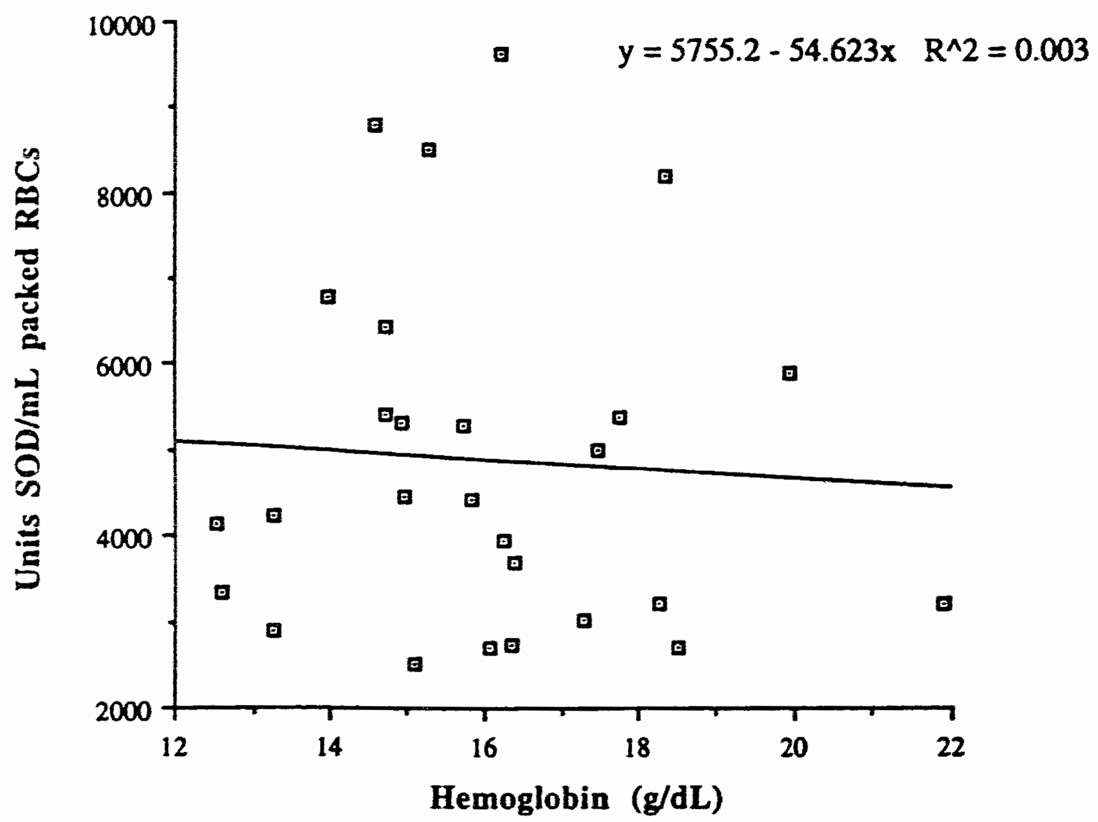


Figure 23. SOD Activity as a Function of Hemoglobin Content in Control Subject's Erythrocytes

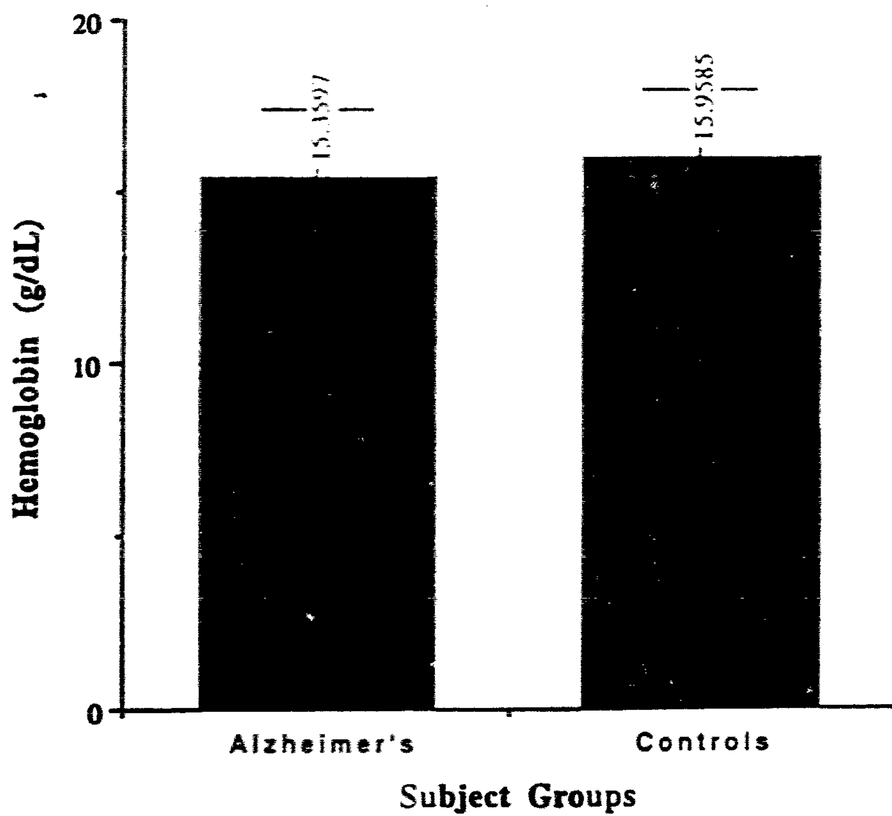


Figure 24. Drabkin **Hemoglobin** Levels **in Erythrocytes** From **Alzheimer's** Patients **and** Control **Subjects**

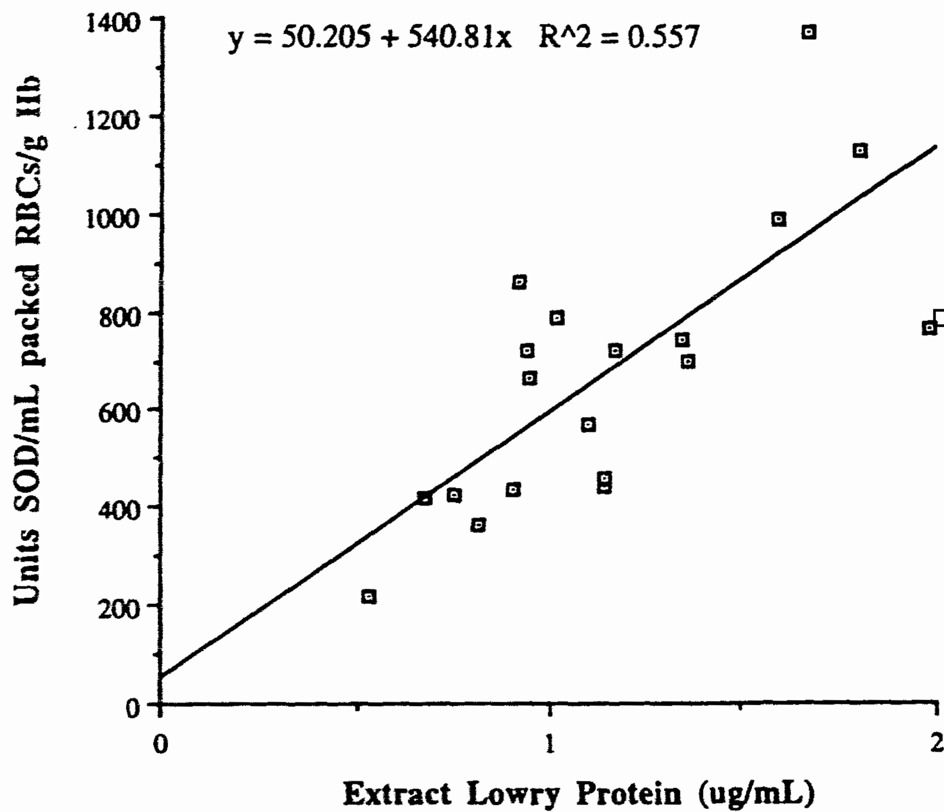


Figure 25. Units of SOD per mL packed RBCs per g Hb as a Function of Extracted Lowry Protein in Alzheimer's Patients

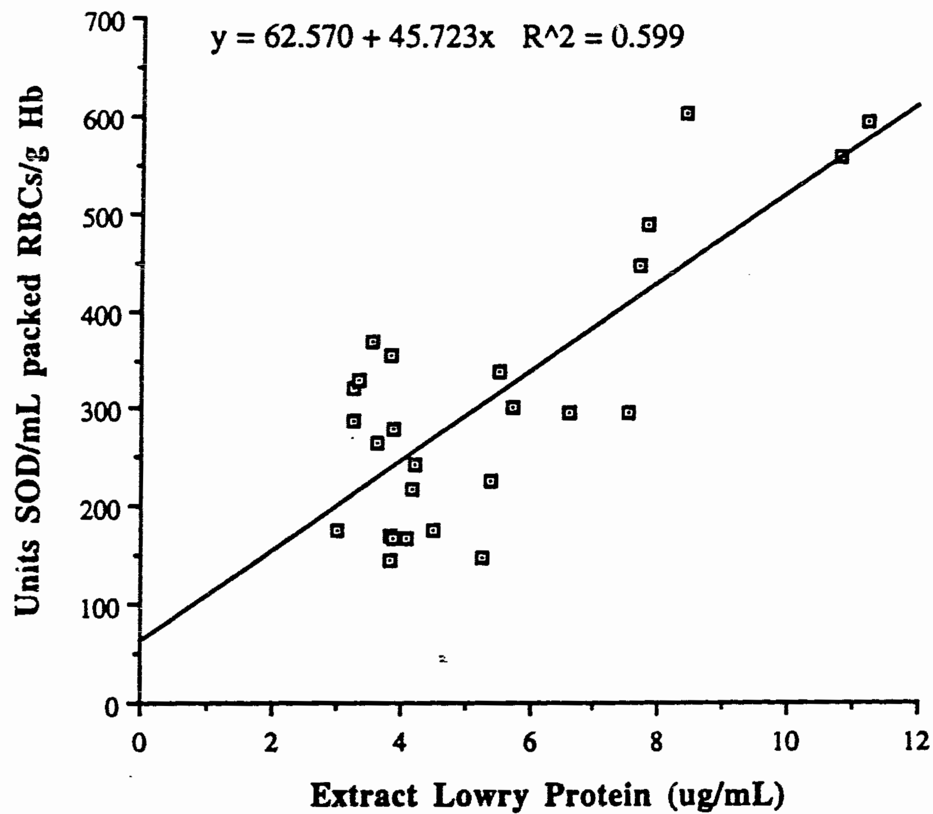


Figure 26. Units of SOD per mL packed RBCs per g Hb as a Function of Extracted Lowry Protein in Control Subjects

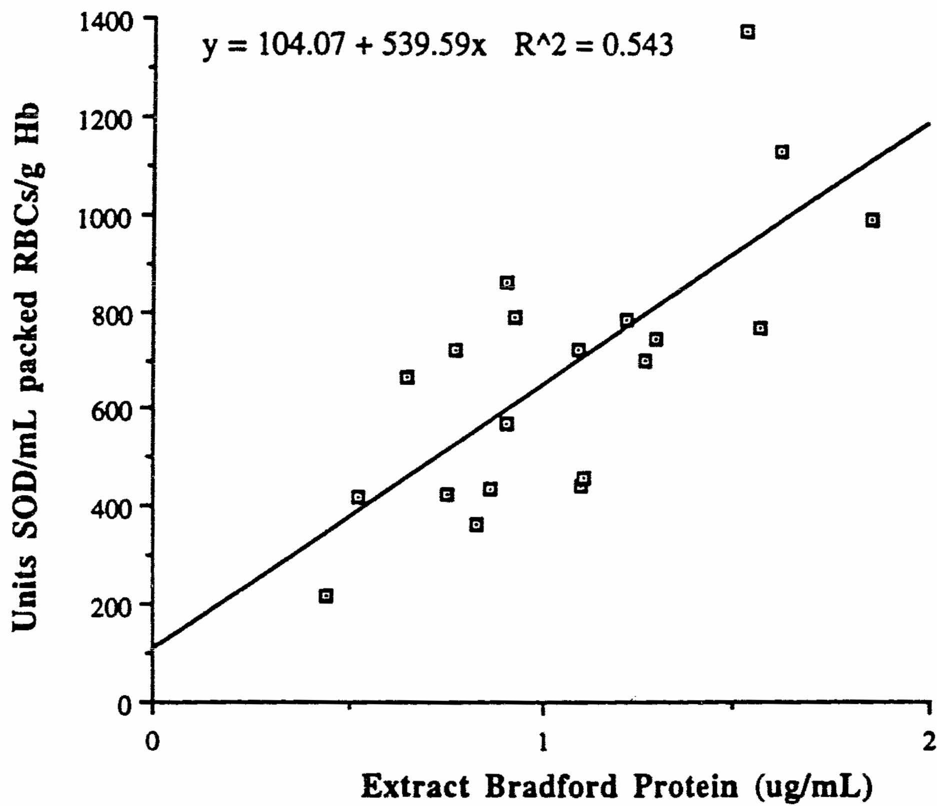


Figure 27. Units of SOD per mL packed RBCs per g Hb as a Function of Extracted Bradford Protein in Alzheimer's Patients

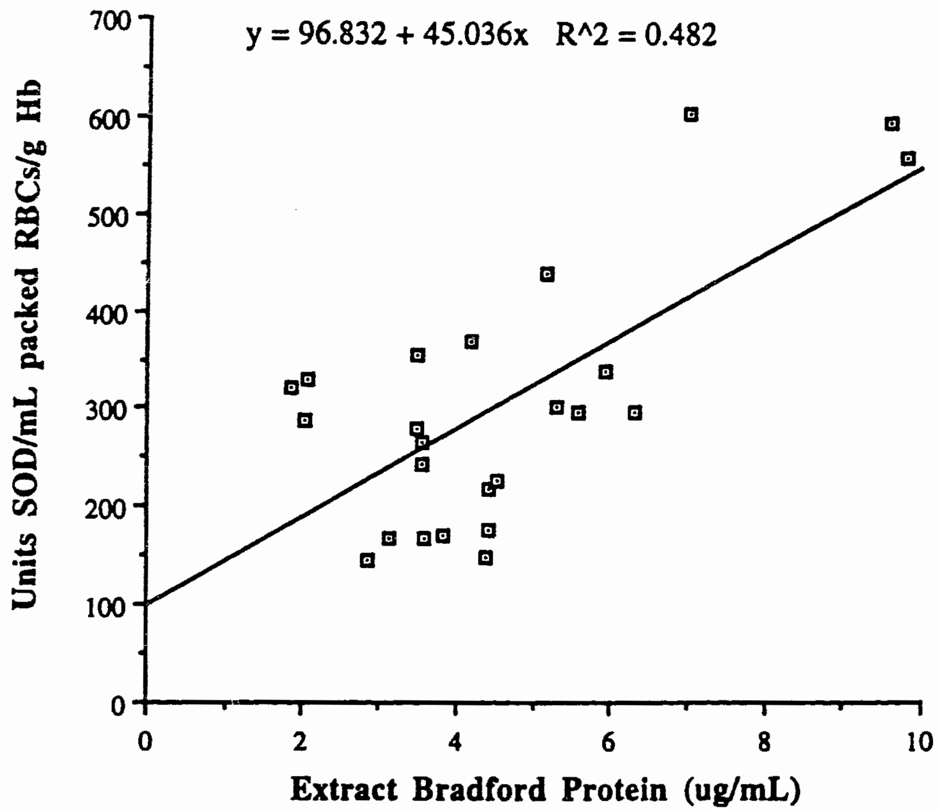


Figure 28. Units of SOD per mL packed RBCs per g Hb as a Function of Extracted Bradford Protein in Control Subjects

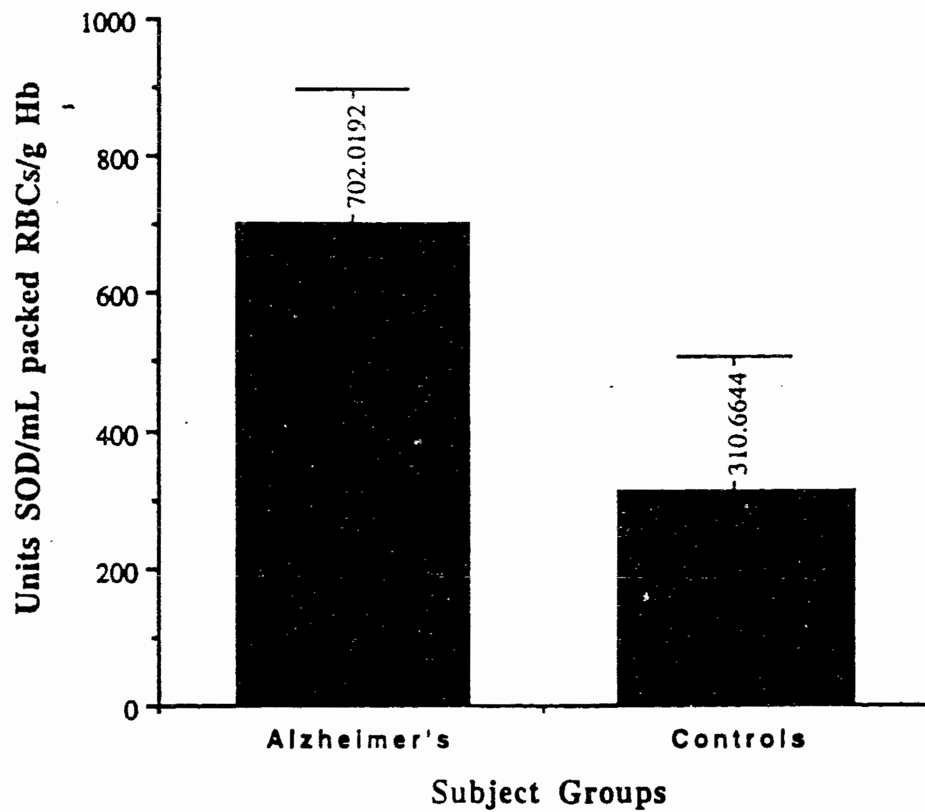


Figure 29. Units of SOD per mL Packed RBCs per Gram of Hemoglobin in Erythrocytes from Alzheimer's Patients and Control Subjects

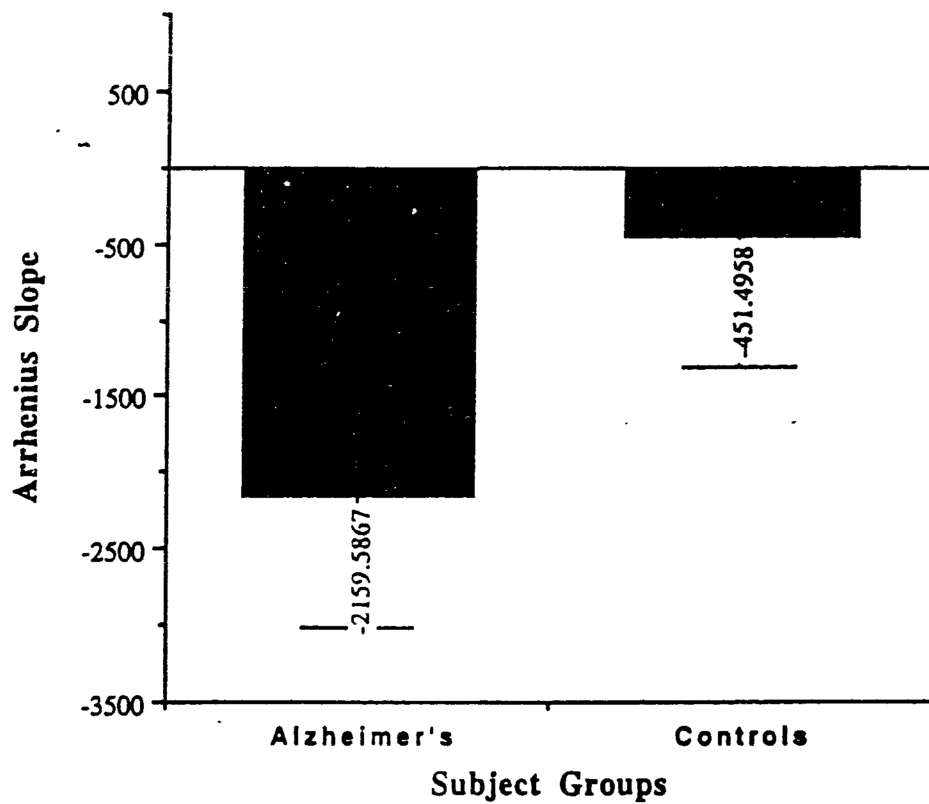


Figure 30. Relative Arrhenius Slopes for Superoxide Dismutase in Alzheimer's Patients and Control Subjects

DISCUSSION

Reports in the literature are conflicting as to what changes, if any, occur in the activity of CuZnSOD in Alzheimer's patients compared to neurologically healthy age-matched adults. These conflicting results are likely attributable to differences in the populations examined, age and sex of the subjects, age classifications, methods of sampling, sample size, the way the samples are prepared, assay methods, conditions of the assay, and definition or representation of activity (Abe et al., 1989). It was hoped that the present study could help elucidate the differences, if they do exist, between CuZnSOD activity in Alzheimer's disease patients compared to age-matched, neurologically healthy controls. The sample size used in the present study was relatively large compared to that utilized by other studies; therefore, the results were less likely to be influenced by chance. Controls were age-matched to minimize extraneous variables from influencing the data as age may affect the enzyme levels in people. Furthermore, whereas other studies employed a one-point determination of the CuZnSOD activity for each subject, obtaining a value for CuZnSOD activity in this study involved gathering multiple points during the assay for each individual in order to define the line for 1/percent inhibition versus 1/volume CuZnSOD extract used in the assay. This allowed for greater accuracy in elucidating the true value of the activity. In addition, total protein content in the chloroform/ethanol/water extracts was determined to monitor possible changes in the specific activity of CuZnSOD. To elucidate whether there were alterations in the physical properties of the CuZnSOD enzyme, a temperature study on the relative rates of the CuZnSOD enzyme was performed at two different temperatures to give an indication of alterations in enzymatic activation energy. The Arrhenius experiment used to detect changes in CuZnSOD that are related to changes in physical properties

of the enzyme was not done in any other studies to our knowledge. Due to the methodology employed in this study, it was possible to ascribe changes in total SOD activity to changes in either catalytic efficiency of the enzyme or to changes in the amount of SOD gene product present.

A graph of relative SOD activity (kcat Et/Km)' versus Lowry (or Bradford) protein in the SOD extracts can be influenced by three different phenomena: (1) Changes in the amount of SOD (Et); (2) changes in the physical properties of the SOD enzyme (kcat/Et); (3) differences in the amount of protein surviving precipitation in the chloroform/ethanol extraction (specific activity of the extract).

Analysis of the graph of relative SOD activity (kcat Et/Km)' versus Lowry (or Bradford) protein revealed a slope which is approximately ten times steeper for the Alzheimer's patients compared to the old, neurologically healthy control subjects. All three of the above mentioned factors could be influencing this graph.

A plot of relative SOD activity versus Lowry protein results in a linear graph for each subject population. This is because the SOD activity is proportional to the total amount of protein extracted within each subject class (Fridovich, personal communications). Both the Lowry method and Bradford method of protein determination, which were performed on the SOD extracts, revealed that the Alzheimer patients had approximately four to five times less protein in their extracts compared to the neurologically healthy age-matched controls. This indicates that the amount of total proteins capable of surviving the chloroform/ethanol extraction was much less than that which survived in the controls. Therefore, the specific activity of the extract from the Alzheimer's patients was higher than that of the controls.

Similarly, results from gel electrophoresis also showed that the Alzheimer patients SOD extracts were much cleaner (i.e., contained less contaminating proteins) than that of the controls by a factor of approximately five. The Alzheimer patients had about 9% SOD purity compared to 1.3 % SOD purity in the control subjects based on calculation of specific activity available from the data collected. A graph of distance travelled on the gel (Rf values) versus 1/log molecular weight (kDa) revealed that only five proteins survived the chloroform/ethanol extraction of the samples; Bands 62, 54, 16, and 14 kDa protein were tentatively assigned as glutathione reductase (63 kDa), catalase (57 kDa), CuZnSOD and hemoglobin (16 kDa; both CuZnSOD and hemoglobin run together), and calmodulin (14 kDa). In the Alzheimer's disease patients the 16 kDa band accounted for approximately 50% of the staining whereas in the controls it accounted for only 15%. The relative intensity of catalase/CuZnSOD was significantly lower in the Alzheimer's disease patients compared to the controls. This suggests that the enhanced CuZnSOD activity observed in the Alzheimer's disease patients is not associated with a parallel increase in catalase activity; this may result in an imbalance in the antioxidant levels with a consequent increase in the hydrogen peroxide levels in the cells.

The drop in protein content observed in the extracts may be attributed to the fact that oxidatively stressed proteins could not survive the chloroform/ethanol extraction procedure, which is a very harsh treatment on proteins. Nevertheless, most of the SOD enzyme survives this procedure as it is an extraordinarily hardy protein. The Alzheimer's patients had a greater drop out rate of proteins due to their having more oxidatively stressed, weaker, more fragile proteins which may have polymerized and precipitated out of the solution. This may be a consequence of cross-linking of proteins to lipid peroxyradicals. Aging has been shown to increase water

insoluble protein precipitate in brain tissue; this has been attributed to free radical cross-linking of general proteins (Nagy et al., 1980). In fact, gross loss of protein solubility due to free radical damage was observed by Nagy et al. (1980).

A comparison of the units SOD/mL packed RBC, which is corrected for dilutions to the blood and is not affected by proteins dropping out in the extraction procedure, demonstrates that the Alzheimer's patients have twice (Alzheimer's patients had 10 798 Units/mL packed RBCs compared to 4 881 Units/mL packed RBCs in the controls) the SOD activity of the neurologically healthy controls. This increased activity may only be caused by two factors: (1) increased expression of the SOD gene (i.e., a greater amount of the enzyme) and/or (2) changes in the physical properties of SOD (e.g., Ea). Note, the large range observed in the amount of SOD units/mL among the subjects is consistent with the findings of other researchers who found a five-fold variation in activities of CuZnSOD, glutathione reductase, glutathione peroxidase, and catalase (Guemouri et al., 1991). This biological variability may be due to the effects of factors which may influence CuZnSOD level such as smoking, alcohol consumption, and intake of certain drugs like anti-inflammatory agents, anti-depressants, and thyroid hormones (Guemouri et al., 1991), or individual metabolic rate.

This finding that Alzheimer's disease patients have significantly elevated (approximately double) CuZnSOD activity compared to age-matched, neurologically healthy controls is in good agreement with results reported by Nichols and Beattie (personal communications), who also found that Alzheimer's disease patients had approximately twice the amount of immunoreactive CuZnSOD, as determined by ELISA, in the Alzheimer's patients erythrocytes compared to the controls. It is also consistent with the findings of Perrin et al. (1990), who found that

Alzheimer's disease patients had significantly higher CuZnSOD levels in their red blood cells compared to age-matched, institutionalized, neurologically healthy controls. The work done by Thienhaus et al. (1986), which demonstrates an increase in CuZnSOD activity in skin fibroblasts from a 53-year-old patient with familial Alzheimer's disease compared to that observed in a normal, healthy 55-year-old man also supports the results of the present study. Another study whose findings were in line with those of the current study is that of Zelman et al. (1989), who examined fibroblast cell lines derived from patients with familial Alzheimer's disease and age-matched controls exhibiting no neurological symptoms. These researchers discovered a significant 30% enhancement of CuZnSOD activity in Alzheimer's disease patients when compared to normal controls.

The results of the present study are in disagreement with those published by Sulkava et al. (1986), who analyzed CuZnSOD activity in red blood cells from four patients with non-familial Alzheimer's disease (age range = 58-74 years old; mean age = 69 $\pm$ 7.4 years) in comparison to that of five healthy controls (age range = 48-64 years old; mean age = 54.8 $\pm$ 7.9 years) and observed no change in erythrocyte CuZnSOD activity between these subject groups. However, these researchers examined only a small series of subjects; therefore, chance may be affecting their results. In addition, age-matched controls were not utilized which likely influenced the findings. Alzheimer's disease patients were on average fourteen years older than the controls.

The results of another study, performed by Jeandel et al. (1989), also differ from the findings of the present study. These experimenters showed no variation in erythrocyte CuZnSOD

activity in Alzheimer's disease patients as compared with controls. Again, this discrepancy with the current study can be explained by the use of non-age-matched subjects.

In order to evaluate the possibility that these changes in SOD activity are due to changes in physical properties or catalytic efficiency, relative Arrhenius slopes of the Alzheimer's patients and the control subjects were compared. The ln % inhibition versus 1/Temperature was plotted, and the slope of this graph gave a reflection of -Ea (enthalpy). The slope of the ln % I vs. 1/T graph can be positive, negative, or zero since there is competition between cytochrome C and SOD for the superoxide radical. If SOD outcompetes cytochrome C, then the slope is negative. However, if cytochrome C beats SOD in capturing the superoxide radical, then the slope of this graph is positive. If both SOD and cytochrome C are equal in their ability to capture the superoxide radical, then the slope of the graph is zero. Because of this competition, there will be variance in the subject groups for this value around the zero point for a bar graph of the mean magnitude of the slopes (of the ln % I vs. 1/T) compared to the subject groups. Furthermore, the standard deviation will be high relative to the magnitude of the mean slope value for each subject category.

It was found that the Alzheimer's patients had a higher activation energy (Ea) for SOD compared to the neurologically healthy controls. This indicates that the Alzheimer patients SOD is inhibited by some form of post-translational modifications compared to the controls. One study has demonstrated that alkylation of CuZnSOD enhances its structural stability (Jabusch, 1980). This may lead to a diminished rate of degradation of the enzyme in Alzheimer's disease patients; thus, resulting in higher concentrations of the enzyme being present in the cells. Another possibility is that the greater stability of the CuZnSOD enzyme might lead to an elevated

activity of the enzyme. The activation energy increase for SOD in the Alzheimer's patients would tend to decrease activity. Increased activation energy would tend to decrease the catalytic efficiency and the rate of the Alzheimer's disease patients' CuZnSOD enzyme. This trend is in the opposite direction to the overall increase in Alzheimer's disease patient's superoxide dismutase activity. Therefore, the two-fold increase in SOD units of activity per mL packed RBCs compared to the controls must be explained only by enhanced expression of the SOD gene and/or decreased rate of degradation of the SOD gene product in the Alzheimer's patients blood. Control of CuZnSOD activity at the pre-translational level of gene expression has been documented in both rat and mouse (Delabar et al., 1987). It appears that increased gene expression of CuZnSOD may be an overcompensation for loss in catalytic efficiency. In Alzheimer's disease patients, there may be an enhanced rate of CuZnSOD synthesis in an attempt to make up for the loss in CuZnSOD activity in their cells due to defective SOD protein.

This situation can be rationalized as a homeostatic mechanism which attempts to regulate the amount of SOD activity in the Alzheimer's patient's blood. It may be that the Alzheimer's patient's SOD is inhibited by post-translational modifications associated with the disease process and the body attempts to compensate for this by increasing SOD gene expression in order to effectively scavenge the superoxide radical. Nevertheless, this enhanced SOD activity may be maladaptive and may lead to further tissue damage. Alternatively, overexpression of CuZnSOD in the Alzheimer's patients could be the primary event which, consequently, may send a signal to a homeostatic mechanism to reduce the maximal velocity or increase Km of the SOD enzyme in an effort to regulate the enzymatic activity.

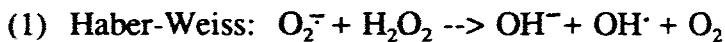
The modification of catalytic efficiency of an enzyme by allosteric mechanisms is well established in enzymology. Specifically, the enzymatic activities of phosphofructokinase, fructose-1,6-bisphosphatase, pyruvate kinase, glucose-6-P-dehydrogenase, cAMP-dependent protein kinase, guanylate cyclase (Ziegler, 1985) and glutathione-S-transferase (Shen et al, 1993) are modified by thiol/disulfide redox regulation. Also, the activity of glucose-6-P-dehydrogenase diminished and the activity of gluatathione peroxidase in red blood cells decreased in response to exposure to hydrogen peroxide in vitro (Vives et al., 1988).

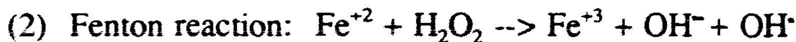
Evidence for toxicity due to overexpression of SOD:

Chemically reactive oxygen free radicals are generated as a normal by-product of oxygen metabolism and after exposure to external agents (e.g., radiation and certain chemicals). These reactive chemical species have been implicated in free radical damage in aging. In 1956, Harman proposed the Free Radical Theory of Aging, in which he states that random deleterious damage accumulates. All living cells which are exposed to oxygen have developed a mechanism to defend themselves from free radical attack. The antioxidant CuZnSOD is one of the primary enzymes involved in this defense.

However, evidence exists suggesting that too low or too high levels of SOD may be detrimental to an organism. There appears to be an optimal level for SOD in tissues. Deviations from this level in either direction are deleterious to the individual. Mao et al. (1993) have demonstrated that the application of SOD as an antioxidant has a bell-shaped dose-response curve. If the concentration of SOD is too high, hydrogen peroxide can increase leading to hydroxyl radical production in Fe^{+2} systems. However, inclusion of catalase with SOD reduces

toxic effects by removing hydrogen peroxide (Mao et al., 1993). Since the role of SOD is to scavenge the superoxide radical, and transform it into hydrogen peroxide, which is later reduced to water and oxygen by the catalase enzyme and peroxidases, a relative increase in SOD activity could result in increased generation of hydrogen peroxide. This elevated hydrogen peroxide concentration could, consequently, lead to enhanced lipid peroxidation and other damage. Subbarao et al. (1990), reported that in vitro basal and iron-induced peroxidation is increased in autopsy samples of Alzheimer's cortical tissue compared with non-demented age-matched controls. If the SOD activity is too high relative to catalase's activity, then hydrogen peroxide can build up. The excess hydrogen peroxide can then participate in any of the following reactions:


(1) Haber-Weiss: $O_2^{\cdot-} + H_2O_2 \text{-->} OH^- + OH^{\cdot} + O_2$


(2) Fenton reaction: $Fe^{+2} + H_2O_2 \text{-->} Fe^{+3} + OH^- + OH^{\cdot}$

(3) Thiol oxidation: $2RSH + H_2O_2 \text{-->} RSSR + 2H_2O$

(4) Lipid epoxides: $RCH{=}CHR' + H_2O_2 \text{-->} RCHOCHR' + H_2O$

Other studies indicating that excessive SOD levels are harmful to organisms includes the study conducted by Bernier et al. (1989). They discovered that SOD at doses of 0 to 8 x 10^4 units decreased reperfusion induced cardiac arrhythmias in rat heart. However, doses of SOD greater

than 8 x 10^4 units increased ventricular fibrillation. Catalase, on the other hand, decreased ventricular fibrillation increasingly well up to the highest concentration tested. Another study demonstrated that human CuZnSOD transfected and overproduced in mouse epidermal cells caused DNA strand breaks, growth retardation, and cell death in response to oxygen metabolites. In contrast, cells doubly transfected with catalase were not harmed (Amstad et al., 1991).

Overexpression of CuZnSOD may upset the steady-state equilibrium of active oxygen species within the cell, resulting in oxidative damage to biologically important molecules. It has been demonstrated that elevated CuZnSOD levels can enhance the cytotoxicity of oxygen free radicals (Finazzi-Agro et al., 1986; Scott et al., 1987; Iwahashi et al., 1988). The increased CuZnSOD activity in Alzheimer's disease patients compared to controls observed in this study could lead to enhanced hydrogen peroxide production.

Further support for oxidative damage in the etiology of Alzheimer's disease:

The increased reactive oxygen metabolites in the blood could impair the structural and functional capacity of the blood-brain barrier, rendering it more permeable to substances; thus, enhancing the ease with which materials cross from the vasculature to the brain tissue. It has been reported that reactive oxygen species increase endothelial transport of proteins which may partly account for the increased blood-brain vascular permeability observed in the Alzheimer's disease patients (Shasby et al., 1985). Furthermore, although the blood-brain mechanisms in the central nervous system serve to exclude reactive metals from contact with neural tissues (Willmore and Triggs, 1991), head trauma, cerebral infarction, and spontaneous intracerebral hemorrhage disrupt the blood-brain barrier and cause red blood cell extravasation followed by

hemolysis and deposition of iron-containing compounds within the lipid-rich brain tissue (Adam, 1975). In addition, red blood cells from Alzheimer's disease patients have different electrophoretic mobility compared to controls (Walter et al., 1992). Also, it has been found that cultured fibroblasts from Alzheimer's patients have increased susceptibility to free radical damage (Tlsco et al., 1992).

Moreover, the elevated hydrogen peroxide levels in the blood, which are a consequence of the high CuZnSOD activity, could result in enhanced diffusion of hydrogen peroxide across the blood-brain barrier, after which it may be involved in peroxidation reactions and, consequently, cause destruction to brain tissues by cross-linking proteins, compromising the integrity of the membrane of cells, and damaging DNA. In fact, inability to repair DNA damage has been implicated as a cause of Alzheimer's disease in brain tissue (Boerrigter, 1992). Also, cross-linking of proteins due to attack by oxygen free radicals may play an important role in deterioration of membrane functions (Nagy et al., 1980).

The human CuZnSOD gene is located on chromosome 21 at region 21q22. This region is present in triplicate in Down's syndrome patients and contributes to the Down's syndrome phenotype including the following characteristics: Severe mental retardation, slow physical development, increased incidence of leukemia, high susceptibility to infections, premature aging, and Alzheimer-type neuronal pathology. Furthermore, the gene for beta-amyloid precursor protein is also found on chromosome 21 (Zubenko and Sauer, 1989). Macrophages have been shown to be responsible for oxidative insult in brain leading to beta-amyloid deposition (Defeudi, 1989). An investigation of the antioxidants on the life span of male LAF mice (Harman, 1968) showed that ethoxyquin (a quinoline derivative) virtually completely prevented the mice from

experiencing spontaneous amyloidosis. Also, free radicals have been postulated to cause helical filament deposition in Alzheimer's disease patient's brains (Zemlan et al., 1989).

Brain cells may be especially susceptible to damage by an altered balance of oxygen free radicals and peroxidative stress, which could result from a relative increase in SOD activity, due to the high concentration of polyunsaturated fatty acids which make up neuronal cell membranes and the very specialized functions these membranes serve. Further exacerbating this situation is the fact that the level of selenium-glutathione peroxidase (which scavenges hydrogen peroxide) activity in neural tissue seems too low to provide protection from peroxide induced lesions. The brain contains relatively low specific activities of hydrogen peroxide-scavenging enzymes (Sinet et al., 1980). This may necessitate increased SOD in the blood to help combat destructive oxygen free radicals.

The brain is particularly vulnerable to oxidative attack and damage for many reasons:

(1) Brain tissue contains very high polyunsaturated fatty acid levels, which are good substrates for peroxidation reactions. Lipids constitute approximately fifty percent of the dry weight of the brain and play a key role in cell structure and function (Strong et al., 1991).

(2) The brain is generally low in oxidative protection when its potential for peroxidation is considered. For example, catalase levels of human brain are extraordinarily low, being less than one percent of that seen in liver or erythrocytes (Marklund et al., 1982). Also, it has been reported that human brain has approximately three percent the total glutathione peroxidase content of human liver (Camagnol et al., 1983). As well, age-associated changes in the composition of phospholipids, consisting of increases in the proportion of saturated fatty acids may be a consequence of increased peroxidation of unsaturated fatty acids (Hegner, 1980).

(3) Brain, especially some areas (e.g., basal ganglia), is highly enriched in non-heme iron, which can participate in Fenton-like reactions (Floyd et al., 1984; Ciuffi et al., 1991; Subbarao et al., 1990). This high iron concentration may influence the potential for peroxidative damage. It has been observed in nerve endings prepared from the neostriatum that iron-induced peroxidation is associated with a diminishment of membrane fluidity and decreased dopamine synthesis (Zaleska et al., 1989). Furthermore, a histochemical examination of brains by Andorn et al. (1990) has revealed that Alzheimer's disease patients have elevated non-heme iron in their brain compared to age-matched controls, particularly in the prefrontal cortex. In addition, increased basal peroxidation, measured by malondialdehyde levels, has been reported to occur in the prefrontal and frontal cortical areas of patients with Alzheimer's disease compared to age matched controls (Andorn et al., 1990; Subbarao et al, 1990). Also, iron-induced peroxidation has been demonstrated to impair high-affinity dopamine uptake in cultured dopaminergic neurons (Michel et al., 1992). These observations of increased basal lipid peroxidation and elevated non-heme iron in areas pathologically affected by Alzheimer's disease supports the concept that enhanced lipid peroxidation plays a role in the expression of the neurodegenerative disease pathology and may be related to alterations in neurochemical parameters associated with Alzheimer's disease.

(4) Brain uses approximately twenty percent of the total oxygen demand of the body.

(5) Brain consists of post-mitotic tissue.

Post-mortem findings in Alzheimer's disease include the following: decreased membrane fluidity, increased protein cross-linking, decreased solubility of membrane proteins, and vacuolarization of neurons (Nagy et al., 1980; Willmore et al., 1981). These changes are also

associated with lipid peroxidation. Lipid peroxidation has been found to be statistically significantly higher in the frontal cortex of brains from Alzheimer's disease patients compared to controls (Subbarao et al., 1990).

Lipofuscin, a heterogenous protein whose synthesis involves oxidative processes, shows an age-related, generally linear accumulation in cells, especially post-mitotic cells such as brain neurons (Floyd et al., 1984). The presence and build-up of lipofuscin in the brains of individuals indicates that the brain is suffering from oxidative stress. It has been demonstrated that raising the level of free radical reactions (e.g., by inducing vitamin E deficiency or increasing the degree of unsaturation of lipid membranes) results in a greater rate of lipofuscin formation in rats (Harman, 1990; Brizzee et al., 1984). If the assault by reactive oxygen metabolites is severe enough, then neurons may die. Since brain cells are post-mitotic cells, this could be potentially harmful, depending on the degree of neuronal cell loss.

Alzheimer's disease patients show excessive neuronal loss in at least four neurotransmitter systems (Mann et al., 1986b; Mann et al., 1989). The loss of neurons in these four systems is associated with pigment formation; lipofuscin in the acetylcholine and serotonin systems and neuromelanin (melanized lipofuscin) in the noradrenaline and dopamine systems (Barden, 1987).

Data supporting the link between perturbation in the ability to eliminate oxygen derivatives and peroxidation of membrane lipids include the following: (1) Hela and L cell clones transfected with a cloned human CuZnSOD gene and containing a 3-6 fold elevated CuZnSOD activity showed an increased in vitro lipid peroxidation; (2) PC12 cell lines expressing the transfected human CuZnSOD gene have shown impaired neurotransmitter uptake resulting from modification of membrane properties of chromaffin granules. This may be secondary to

lipid peroxidation. It was also discovered that the PC12-SOD clones had an impairment in their biosynthesis of prostaglandin E_2, an unsaturated lipid derived from membrane-bound arachidonic acid, a known substrate for lipid peroxidation (Groner et al., 1992). It appears that increased SOD activity is causing alterations in structural and functional elements responsible for neurotransmission efficiency and; therefore, may contribute to neurobiological abnormalities found in Down's Syndrome and Alzheimer's patients. Perhaps the levels of antioxidant enzymes must be maintained in a stoichiometric balance to achieve, or maybe even lengthen, an individual's maximum lifespan potential. With regards to the possible role of SOD and free radicals in neuronal pathology, it is noteworthy that loss of neurons in aging, Down's Syndrome, and Alzheimer's disease patients brains is selective and follows the same pattern and that a deregulation in the activity of the cellular antioxidant system is often implicated (Andorn et al., 1990).

Because Alzheimer's disease is primarily viewed as a brain disease, it could not have been predicted at the outset that anti-oxidant enzymes would necessarily be elevated in the blood of the patients. The fact that CuZnSOD activity is increased in the blood of Alzheimer's patients may indicate that the initial causative event may be an organism-wide increase in oxidative stress. Alternatively, the initial oxidative injury may be restricted to the brain, but due to the limited capacity of the brain to combat oxidative stress, red blood cells (with their antioxidant defenses such as SOD) may be recruited to remove oxygen radicals which diffuse out of the neuronal tissue.

CONCLUSIONS

The following conclusions may be drawn regarding erythrocytes from Alzheimer's patients compared to control subjects:

(1) Alzheimer's patients appear to have enhanced synthesis and/or decreased degradation rate of CuZnSOD.

(2) The proteins in the red blood cells of Alzheimer's patients are weaker and more damaged. This increased fragility of proteins is likely due to enhanced oxidative stress and protein cross-linking occurring in the erythrocytes of Alzheimer's patients.

(3) The CuZnSOD enzyme is more inhibited (by post-translational modifications) as its catalytic efficiency is diminished.

The increased CuZnSOD activity (due to increased synthesis and/or decreased degradation rate of the enzyme), but elevated energy of activation of the enzyme may be a consequence of a compensatory mechanism designed to regulate CuZnSOD activity.

The enhanced CuZnSOD activity in Alzheimer's patients could lead to increased hydrogen peroxide production. If mechanisms which remove hydrogen peroxide are not similarly elevated, hydrogen peroxide may build up. The hydrogen peroxide molecules could cross the blood-brain barrier. Excess hydrogen peroxide could lead to the generation of hydroxyl radicals through Haber-Weiss and Fenton reactions. Since hydroxyl radicals are very reactive and non-speci in their action, they could damage brain regions. If the assault by reactive oxygen metab is severe enough, then neurons may die. Since brain cells are post-mitotic cells, this could be potentially harmful, depending on the degree of neuronal cell loss.

FUTURE STUDIES

In the future, catalase activity should be measured in Alzheimer patients and control subjects to determine how catalase activity varies relative to SOD activity. In addition, a radioimmunoassay should be performed to elucidate the immunoreactive concentration of SOD in order to determine how much increased expression of the SOD gene contributes to the elevated SOD activities found in Alzheimer disease. In addition, the temperature study of CuZnSOD should be repeated over a broader range of temperatures to provide more points on the Arrhenius plot in order to increase the statistical accuracy of the results.

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Appendix A

Aging and Alzheimer's Disease

The age-dependent increase of the prevalence of AD may be better understood in the context of some of the proposed evolutionary aspects of the aging process itself. The degenerative changes that are seen during senescence are ultimately the consequence of an organisms failure to preserve the functional integrity and viability of its organs, tissues, and cells, possibly due to some extent to a decreased accuracy (of the regulation) of macromolecular synthesis (Orgel. 1963, 1973; Kirkwood et al., 1984; Vijg, 1990). The evolvement and improvement of maintenance and repair processes during evolution are thought to be responsible for the rapid increase in life span of higher organisms, i.e., mammals (Sacher, 1982). The selection of such advantageous phenotypic changes may be achieved by increasing the efficiency of some specific enzymes, especially those enzymes involved in maintaining the integrity of DNA (Turturro and Hart, 1991).

An accumulation of DNA damage due to the disinclination of the cell to invest enough energy in mechanisms counteracting the build-up of pernicious DNA damage might thus be considered as one of the major and fundamental causes of aging (Vijg and Knook, 1987; Kirkwood and Rose, 1991). One major age-related defect that might be due to the accumulation of germline mutations in one or more genes is neuronal degeneration.

Appendix B

Age-related Neuronal Changes

Numerous alterations occur in the neurons of the brain with advancing age. The brain loses neurons, and therefore loses weight, as it ages. It has been estimated that there is approximately a 15% reduction in brain weight by the age of eighty (Cote, 1981). However, the brain has a great deal of reserve capacity and despite the age-related loss in neurons, the older brain may lose rather little in functional capacity (Labouvie-Vief, 1985). Four prominent changes have been observed in the brains of aged humans: the accumulation of lipofuscin, granulovacuolar particles, neurofibrillary tangles, and senile plaques.

Lipofuscin is a yellowish-brown pigment. As individuals age, more and more deposits of lipofuscin accumulate in the neurons of specific parts of the brain (Bondareff, 1985). Interestingly, it has been shown that individuals who suffer from vitamin E deficiency display above-average amounts of lipofuscin (Cohen, 1988).

Granulovacuolar particles are small granules of matter in the soma and dendrites of degenerating neurons, especially in the hippocampus. The origin, effects, and functions of these particles are still unknown (Cote, 1981).

Neurofibrillary tangles are one of the most common and dramatic histological changes in the brains of the elderly. These tangles are most concentrated in the cortex and the hippocampus (Reisberg, 1981). Long, straight, thread-like fibers called microtubules exist within neurons which function to transport chemical substances between the distant parts of the neuron. With age, these microtubules become twisted and distorted and progressively fill the soma and push

the nucleus to one side (Rybash, 1985). The presence of tangles may ultimately lead to the dysfunction and death of neurons.

Senile plaques are extracellular spherical masses of a substance called amyloid (i.e., beta amyloid protein). With aging, these plaques increasingly appear between neurons. The amyloid forms a core which is surrounded by degenerating axons and dendrites (Cote, 1981). They are frequently found around synapses and can interfere with normal neuronal communication. Plaques, like tangles, are most likely to be found in the cortex and hippocampus. It has been suggested that the increased incidence of plaques and tangles may be related to memory loss among the elderly (Scheibel and Wechsler, 1986). Recent evidence (Whitson, Selkoe, and Cotman, 1989) suggests that the production of amyloid may represent a neuron's attempt to defend itself against the degenerative effects of aging. Blessed et al. (1968) have found that there is a quantitative relationship between the number of senile plaques and the severity of dementia.

Appendix C

Why do humans live past their age of optimum health and vigor and into senescence?

Aging does not appear to be a natural phenomenon, rather it may be an artifact of the advent of civilization (Cutler, 1984). It does not appear to confer any biological advantage. Some people believe that aging exists to eliminate the old people from the population. However, if aging did not exist, there would be no old people to remove. Others suggest that aging exists to make room for young individuals so that new, fresh, and different ideas, skills, and abilities can be introduced; thereby, providing a foundation from which evolution can take place. However, exogenous environmental pressures and hazards have always been sufficient for this purpose, if indeed making room for younger individuals was evolutionarily beneficial. Thus, aging would not be necessary. Still, others propose that "old" individuals are valuable because they have experience and wisdom which they can pass on to "younger" individuals. "Old" individuals are, therefore, needed to teach and train "young" individuals. This argument again fails because this advantage is one which arises from the individual living a long, healthy life (i.e., from its longevity), not from the fact that he/she ages.

Appendix D

Evolutionary Considerations

The differentiated state of a cell is a highly ordered state. Thus, it is likely that a positive evolutionary force, in which energy has been required to maintain the ordered state and prevent the natural move towards entropy, has driven cells towards such a condition. With the evolution of multicellular organisms, an increased stability of the differentiated cells must have evolved simultaneously. Thus, in order for evolution of multicellular organisms to occur, at least two equally important changes had to take place (Cutler, 1979): (1) Changes in gene expression patterns of cells (which allowed a single fertilized egg to develop into a fully developed animal); (2) Development of means to maintain cells in their proper differentiated state once the genetic program of development had been completed. Many different strategies could have evolved to achieve this aim. Among these are the fast turnover of cells where environmental hazards are great, DNA repair systems, and protection against oxygen free radicals (Cutler, 1979).

If the natural tendency of cells is to move towards a random, dysdifferentiated state, and maintenance of the proper differentiated state of cells extracts a biological cost, then organisms likely evolved a mechanism to maintain the stability and integrity of their cells only to the extent that a biological advantage was endowed by doing so. In other words, a balance between costs and benefits of stability was made. Cells from longer-lived species would be expected to have a more efficient method of maintaining the differentiated state of its cell. However, this difference in stability would not be in excess, but rather just sufficient to prevent aging (if indeed aging occurs through the dysdifferentiation of cells) of the organism to become a significant factor limiting its life span in its natural ecological niche (Cutler, 1978). Thus, the stability of

the proper differentiation of cells as a function of time would be expected to be proportional to the species' maximum life span. Such a correlation, especially within the mammalian species has been found (Cutler, 1978).

Thus, an organism is created through an ordered differentiation of cells, this differentiated state of cells is maintained to an extent that it incurs a biological advantage to the organism and ensures its survival and evolutionary success of the species, after which time the slow, random loss of cellular function and differentiation begins to set in and reduce the health and vigor of the organism. Since for the majority of the evolutionary history of life, animals living in the wild died due to natural hazards in their environment (e.g., predators, climate, disease, etc.), aging played little role in determining the life span of animals and such a decline in optimum functioning and viability of an organism would only be able to manifest in those organisms which live beyond their natural evolved life span (Cutler, 1984).

Appendix E

The Biological Nature of Aging

Theoretically, the aging process can be a result of "active" or "passive" processes. According to the "active" hypothesis, aging has evolved for the good of the organism or the good of the species as a whole. It postulates that aging is an integral part of the genetic developmental program and believes that in the same way that alterations in gene expression result in the development of an organism, further changes in gene expression continue to occur after sexual maturity is achieved, resulting in the aging of the individual. The "active" hypothesis of aging suggests the existence of specific "aging genes" which function to age the organism for the benefit of the individual or the species. Without these genes, aging would not occur.

However, there is no evidence for the existence of such "aging genes". Instead, there are a number of arguments refuting their existence (Cutler, 1984). Firstly, no evidence exists suggesting that aging evolved. Rather, it is more consistent with evolutionary theory that longevity evolved, where the preservation of life has always been sought, and, consequently, this has led to the evolution of anti-aging processes. Secondly, animals living in their natural habitats or ecological niches seldom live to an age where any significant degree of senescence occurs. Practically all animals in the wild are killed by their natural predators or other hazards well before they even begin to show noteworthy declines in performance due to aging. For instance, *Homo sapiens* have existed on the earth for at least 15, 000 years and during most of this period of existence, the average human life span was only about 20-40 years. Because these humans were biologically equivalent to modern day man, their aging rate was in all likelihood similar. Thus, for most of human history, the human population consisted of "young" individuals, where

individuals seldom had an opportunity to grow "old" and show the effects of aging. Thus, there would be little incentive in terms of evolutionary selective pressure to evolve special genes for the purpose of removing individuals from the population. Thirdly, if prolonged life span were truly detrimental, it is unlikely that such a slow and prolonged mechanism, which can extend up to one half the individual's life span, would have evolved for terminating life. Instead, a genetic program of death rather than senescence would have evolved where the individual enjoys optimum health and vigor for most of his/her life, and then, at a certain age, he/she would die.

If aging is not "active" in nature, the remaining possibility is that it is a "passive" process (Cutler, 1984). That is, the cause of aging must lie in the basic make-up and functions of an organism which enable it to stay alive and live as long as it does in good health. The passive hypothesis postulates that aging is caused by reactive by-products of normal, vital metabolic life processes. Normally, these by-products are not significantly damaging to the organism, but if the organism lives longer that it was evolved to live (i.e., it is removed from its ecological niche and placed in a much safer environment), then, with the passage of time, these reactive by-products become detrimental to the organism and cause it to age. Thus, the cause of aging is pleiotropic in nature. That is, aging is a result of normal biological processes essential for life, but which also have long-term negative effects on the organism.

Appendix F

The Pleiotropic Nature of Oxygen

"The world is full of mysteries. Life is one." - J.B.S. Haldane

The concept that a portion of the atmosphere matters greatly to life is very old. This essential element was discovered to be oxygen. Oxygen is the third most abundant element in the universe, being surpassed only by helium, which is chemically unreactive, and by hydrogen. Unless the temperature is below -183 C, oxygen is a gas. To be a good energy source, a substance must have a high potential, but also it must react slowly. If it reacted rapidly, it could not store the energy. There are other species which have a higher potential than molecular oxygen, such as fluorine, ozone, atomic oxygen, and chlorine, but molecular oxygen is sluggish in reacting (Gilbert, 1960). Thus, oxygen is a good energy source as it is abundant, easily available, has a high potential, and reacts slowly.

When the earth was formed about 4.5 billion years ago, the atmosphere was reducing. The universe is predominately hydrogen. Hydrogen is escaping very slowly from the earth, making this planet a pinpoint of oxidation in a reducing universe. The biosphere was born in a reducing environment and slowly developed the capacity to produce oxygen by photosynthesis. The crust of the earth contains a very large predominance of oxygen. Since oxygen is reactive, it would not remain in the free molecular form very long on a geological time scale. The only reason molecular oxygen is present on earth is that the biosphere rapidly produces it.

Our planet's atmosphere contains approximately 20% oxygen, an amount of this vital substance which has made life possible in all its manifestations. To what extent life as we know it on earth has developed in order to accommodate to this particular oxygen tension, and to what extent life would have developed under conditions of lower or higher oxygen tensions are a matter for speculation. In fact, it has been said that we exist on Earth due to an accident of quantum mechanics. This is that oxygen does not consume us instantly as the thermodynamic driving force of free energy insists that it will. A barrier to this reaction, traceable to delicate interelectronic interactions in the oxygen molecular anion, O_2, allows us to enjoy our brief lifetime on Earth in productive endeavors.

Some organisms, such as anaerobic bacteria, cannot tolerate the oxygen present in air and thrive only when oxygen is sparse or absent. However, the great majority of animals and plants have adapted to live under existing atmospheric conditions; thereby, taking advantage of oxidative processes which produce much greater amounts of energy per metabolite used than glycolysis or other half-reactions implemented by many lower organisms or by higher organisms under times of stress. Nevertheless, oxidative reactions are associated with potentially harmful effects on numerous reactions and enzymes in the cell. Thus, during evolution, it was necessary for animal and plant cells to develop special mechanisms to control the oxidizing power of molecular oxygen.

During the interval from 3800 to 2000 million years ago, iron acted as an oxygen sink (Cloud, 1976). The single cell organisms used the highly efficient respiration, and any of the photosynthetic oxygen produced which leaked out of the cell would either be taken up by other

neighbouring cells or by the reduced iron. Thus, the reduced iron acted as an external antioxidant to the biosphere.

However, during the transition from an anaerobic to an aerobic environment, the biosphere necessarily developed antioxidant defenses, as it experimented with a more productive energy source. Cloud (1976) believes that the reason it took 1.8 billion years from the time that oxygen was liberated by photosynthesis to the time that free oxygen appeared was that this time was necessary to build up the necessary antioxidant defenses. Thus, there was an evolutionary pressure for the development of antioxidant defenses (Gilbert, 1960; Gerschman, 1964). This reasoning seems to be correct since oxygen is very toxic (Gerschman, 1964).

Appendix G

Historical Perspective of the Dual Nature of Oxygen

> "Poison is in everything, and no thing is without poison. The dosage makes it either a poison or remedy." - Paracelsus, 16th century

Oxygen is an energy source par excellence. Oxygen gave the biosphere a greater mobility and permitted the appearance of multicellular organisms. However, there is a price to pay for this mobility, and it is that the biosphere has to cope with oxygen toxicity. To combat this toxicity, during evolution, living matter had to develop adequate antioxidant defenses in order to resist the destructive capability of oxygen in air. Thus, antioxidants of a varied nature were developed by the biosphere. However, this defense can never be perfect, and so the biosphere still has to suffer from oxygen toxicity. Thus, we see here the dual effect of oxygen, that is, life-giving energy for aerobic organisms and destructive influence on all forms of life.

Uncontrolled oxidations from a continuous small "slipping" in the defending system could be an important factor in the process of aging and also a factor contributing to life span. Antioxidant defenses can inhibit the destructive tendency of oxygen, but can not completely prevent it from occurring. This is the price that the biosphere pays for its existence.

Fire and life have intrigued mankind for over 2500 years. From ancient times, an analogy between fire and life has been made. The independent discoverers of oxygen, Priestley and Scheele, were aware of this analogy and recognized that oxygen, like fire, could be not only beneficial, but also dangerous.

In the second half of the 18th century, both Priestley and Scheele performed experiments with this newly isolated oxygen on both combustion and respiration. Priestley (1775) compared these two processes, stating:

> "(oxygen) might not be so proper for us in the usual healthy state of the body: for, as a candle burns out much faster in dephologisticated (oxygenated) than in common air, so we might, as may be said, live out too fast, and the animal powers be too soon exhausted in this pure kind of air. A moralist, at least, may say, that the air which nature has provided for us is as good as we deserve."

The first report of oxygen toxicity was given by Scheele, who stated that "Plants (peas), however, will not grow noticeably in pure fire air (oxygen)" (Scheele, 1777). In 1777, Macquer stated:

> "If the atmospheric air were perfectly pure, the life of animals breathing it would be much more energetic, better, and more pleasant in many ways; but at the same time it might be proportionately shortened, and being rapidly consumed by such active air, they might live only one quarter of time that they live in the ordinary air of our atmosphere, impure that it may be."

Although shortly after the discovery of oxygen, some physicians began using oxygen as the cure-all for many illnesses, within a few years after the introduction of oxygen therapy, the medical profession became extremely skeptical of this treatment. In fact, at the beginning of the nineteenth century, the medical profession was generally aware that oxygen can be toxic.

Although the dangers of oxygen were recognized as soon as oxygen was discovered, it was Paul Bert (1878) who put oxygen toxicity on a firm experimental basis. Bert demonstrated in his pioneering studies, a century after the discovery of oxygen, that all living organisms are subject to oxygen toxicity. Shortly before, Louis Pasteur had shown that the mere presence of oxygen could kill certain organisms.

Bert exposed animals, plants, and microscopic organisms to various oxygen pressures under different total barometric pressures. His results show that, "The whole aggregation of living beings, in a word, dies absolutely when the oxygen tension is high enough." He observed that elevated oxygen pressures result in "violent convulsions displayed by the higher animals." Bert stated that: "The organisms at present existing in a natural state...are acclimatized to the degree of oxygen tension in which they live: any decrease, any increase seems to be harmful to them when they are in a state of health."

Appendix H

Antioxidants

Uric Acid

Uric acid, a waste by-product of purine metabolism, has been found to be an excellent antioxidant which is capable of protecting membranes from lipid peroxidation (Ames, 1981). Studies have been conducted to evaluate uric acid's role as a potential longevity determinant. Tissue levels of uric acid correlate well with species' MLSP and LEP values (Cutler, 1986). The data indicates that the unusually high levels of urate (the sodium salt of uric acid) in piasma and tissue in humans is playing an important biological role as an oxidant in the high LEP value in humans.

A study of the biochemical mechanisms determining urate levels in tissue and plasma have revealed that only a small number of genetic regulatory changes need to take place to account for the evolutionary increase in urate concentration in primates with increasing MLSP (Cutler, 1984). Serum levels of uric acid, which has been proposed to be a major metabolic antioxidant, have been reported to be reduced in Alzheimer's disease patients (Kasa et al., 1989).

Carotenoids

Carotenoids, synthesized only in plants, are believed to play an important role in protecting plants from free radicals produced during photosynthesis. Beta-carotene, a member of the carotenoid family found in all tissues of the body and a precursor to vitamin A synthesis, is thought to have excellent antioxidant properties (Krinsky and Deneke, 1982). It is also of

pharmacological interest in the prevention of cancer since it has been found that people with low levels of tissue beta-carotene are unusually susceptible to a variety of different cancers (Peto et al., 1981). A good correlation was found between carotene levels and MLSP, but the correlation was not statistically significant for vitamin A in species whose MLSP was greater than approximately twenty years (Cutler, 1984).

Alpha-tocopherol (Vitamin E)

Alpha-tocopherol or vitamin E is a well known tissue antioxidant (Balin et al., 1978). Human serum has an unusually high serum alpha-tocopherol concentration compared to species with a shorter life span. There is an excellent positive correlation between vitamin E levels in plasma as a function of both the species' MLSP and LEP values (Cutler, 1984). Experiments studying oxidative changes occurring in vitamin E deficient and aging rats have demonstrated increases in brain lipid peroxides, with large amounts occurring in the olfactory bulb (Noda et al., 1982), and an accumulation of lipofuscin (a lipid-derived oxidation product), especially in the hippocampus (Sarter and Van Der Linde, 1987), which are brain regions of particular significance in Alzheimer's disease. A reduction in serum levels of vitamin E (Jackson et al., 1988) and accumulation of neuronal lipofuscin (Dowson, 1982) in Alzheimer's disease have also been observed.

Appendix I

Metals and Alzheimer's Disease

Aluminum

Aluminum has been identified within diseased neurons and is a consistent feature at the core of senile plaques; co-localized with silicon as amorphous aluminum silicate (Katzman, 1986). A positive correlation between high aluminum content in drinking water and the incidence of Alzheimer's disease has been reported (Martin, 1989). Furthermore, neurotoxic properties of aluminum have been elucidated. For example, aluminum inhibits the hexokinase reaction in the metabolism of glucose involving ATP-Mg. In vitro, aluminum binds to ATP ten times more readily than Mg^{+2}, thus displacing it to form inactive ATP-Al, thereby inhibiting the reaction. Under normal cell conditions, citrate binds to aluminum twenty times more readily than ATP, thus preventing the formation of ATP-Al. However, if excessive aluminum is present, the inhibition of the ATP-Al complex is impaired, leading to abnormal metabolism. Normally, aluminum is exclusively bound to transferrin, a plasma glycoprotein, with very little free aluminum in circulation. The release of aluminum from transferrin depends upon the presence of transferrin receptors on the cell surface. Once bound to the receptor, transferrin becomes incorporated into an endosomal compartment within the cell which has a sufficiently low pH to cause dissociation of aluminum from transferrin. The brain has very few transferrin receptors, thus, normal brain aluminum uptake is very low. An impairment of aluminum binding to transferrin due to oxidative stress could increase the amount of free circulating aluminum, consequently increasing the risk of neurotoxicity by increasing aluminum deposition in the brain.

Calcium

A microtubule-associated tau protein has been identified in the paired helical filaments of neurofibrillary tangles (Wischik et al., 1988). Impairment of microtubule function has been hypothesized to play a key role in Alzheimer's disease pathogenesis (Evans et al., 1990). Reactive oxygen metabolites induce the release of intracellular calcium from bound reticulum vesicles (Trimm et al., 1986) and act synergistically in injuring brain synaptosomes (Braughler et al., 1985). Reactive oxygen also increases endothelial transport of proteins, a finding relevant to the enhanced blood-brain vascular permeability in Alzheimer's disease.

Iron

Non-haem iron in the prefrontal cerebral cortex has been shown to be increased in Alzheimer's disease subjects (Andorn et al., 1990). Available or 'catalytic' iron is of direct relevance to the capacity of iron to catalyze hydroxyl ion free radical production (Gutteridge and Halliwell, 1987), the hippocampus being susceptible to iron-mediated lipid peroxidation (Zaleska and Floyd, 1985).

Zinc

Researchers have found that human serum zinc levels show an age-related decrease (Lindeman et al., 1971); this is partly due to a reduced dietary intake of zinc with age (Greger, 1977). Neutron activation analysis for trace elements of serum (Corrigan et al., 1987) and brain (Ward and Mason, 1987) has determined a deficit of zinc with an accompanying increase in aluminum and calcium levels in the hippocampal tissue in Alzheimer's disease subjects (Burnet,

1981). Trace metals (Prohaska, 1987), particularly zinc, play an important role in neurobiology as they are involved in synaptic transmission (Hesse, 1979). Binding of zinc by metallothionein-like proteins in the brain (Ebadi and Hama, 1986) may be of relevance for the protection of tissue against the effects of oxidative stress induced by free radicals (Thornalley and Vasak, 1985).