Order Number 1350790

Vitamin E turnover in cultured pulmonary alveolar macrophages

Hoeger, Glenn Charles, M.S.

The University of Arizona, 1992

U·M·I

300 N. Zeeb Rd.
Ann Arbor, MI 48106

Vitamin E Turnover in Cultured Pulmonary Alveolar Macrophages

by

Glenn Charles Hoeger

A Thesis Submitted to the Faculty of the

DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

MASTER OF SCIENCE
WITH A MAJOR IN TOXICOLOGY

In the Graduate College

THE UNIVERSITY OF ARIZONA

1992

STATEMENT BY AUTHOR

This thesis has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgement of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his or her judgement the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: _____

APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

Daniel C. Liebler
Assistant Professor of
Pharmacology and Toxicology

10-8-92
Date

Dedicated to Kimberly for her love and support.

ACKNOWLEDGEMENTS

It is impossible for me to completely express my gratitude to all the people who have aided me during these past three years. I want to start by thanking Dr. Daniel Liebler for the time, guidance and friendship which he extended to me while working in his lab. I would also like to express my most sincere appreciation to Dr. Dean Carter and Dr. Clark Lantz for their contributions to my education and their infinite patience. In addition, I want to take this opportunity to thank Dr. Paul St John for the use of his culturing facilities and technical support. Finally, I would like to thank Dr. Raphael Gruener for giving me a chance to pursue a career in science.

To Jeanne Burr, Amy Sharpe, Steve Stratton, Richard Gustafson, Kevin Divine, and Dwayne Hill, I want to thank all of you for making my time with the Pharm/Tox department extremely enjoyable and productive. You've all had a hand in the progress of my research and I will always think of you when I reflect back on these times.

I want to thank my parents for all the years of unwavering support, their faith in me was often more than I had in myself. I know this is as much their achievement as mine. Finally, I want to thank my wife and closest friend, Kimberly. For all we've been through-thank you, I love you.

TABLE OF CONTENTS

PAGE

LIST OF FIGURES

ABSTRACT

Vitamin E (α-TH), the primary lipid soluble antioxidant, can protect tissues from oxidative insult. Oxidant-producing pulmonary alveolar macrophages (PAM), may depend on α-TH to prevent oxidative damage. α-TH levels in cultured PAM declined rapidly during the first 12-18 hours in culture. Approximately 60% of the decrease was detected as unoxidized α-TH released to RPMI 1640 (containing 5% fetal bovine serum (FBS)) culture medium. α-TH was not detected in serum-free Ham's F12 medium. PAM appeared to reabsorb α-TH from the medium. PAM activation with phorbol myristate acetate (PMA) did not affect cellular α-TH depletion. However, the amount of α-TH detected in the medium of PMA treated cultures was only 50% of that detected in medium from untreated controls. Inhibition of superoxide production with iodoacetate had no effect on cellular depletion kinetics, however medium α-TH levels were still 50% of controls. Inhibition of nitric oxide synthesis appeared to have no effect on α-TH status.

CHAPTER 1

INTRODUCTION

OVERVIEW

Free radicals have been increasingly implicated in a wide variety of disease states and mechanisms of tissue injury. Many prominent diseases such as cancer, atherosclerosis and rheumatoid arthritis may develop through theorized mechanisms involving free radicals, while tissue damage associated with ischemia and trauma also lend strong evidence to the participation of radicals. Free radicals are continuously being produced by a large number of sources. Exposures to environmental pollutants, ionizing radiation, chemical toxins and certain drugs can lead to free radical production *in vivo*. Organisms themselves are also responsible for radical formation through cellular respiration and immune responses. Oxygen metabolism, the driving force behind cellular energy production, is a chief source of superoxide ($O_2^{\cdot}$) radicals. Highly oxygenated tissues, such as the lungs, can be placed under considerable oxidative stress by such metabolism. Pulmonary alveolar macrophages (PAMs), the lung's resident phagocytes, can add to this oxidative stress by generating $O_2^{\cdot}$ as a portion of their immune response. As a result, oxygen derived free radicals present the lungs with an oxidative challenge that constantly must be defended against.

Antioxidants are a group of compounds and enzymes which are responsible for the protecting organisms against oxidative injury. This group consists of an assortment

of molecules with very different mechanisms of action. Some antioxidants operate by inhibiting radical formation. They metabolize radical precursors, such as hydrogen peroxide, or act to sequester catalytic metals. Other antioxidants such as vitamin C, ß-carotene and vitamin E are able to intercept radicals that already have been generated. All these antioxidants work in concert to maintain a steady state with respect to oxidative damage.

The level of oxidative stress to which tissues are exposed has been of interest to many researchers. Measurement of oxidative stress by analysis of damaged DNA bases, protein oxidation products and lipid peroxidation products have all been investigated. An alternate approach to evaluate the effects of oxidative stress is to measure antioxidant function rather than oxidative damage. Although the levels of tissue antioxidants such as glutathione, vitamin C and vitamin E may be changed by oxidative stress, antioxidant levels themselves may depend on factors other than oxidative stress. The ability of tissues to replace or regenerate antioxidants implies that antioxidant turnover, rather than antioxidant levels, may better reflect oxidative stress than antioxidant levels. This point is illustrated by studies of glutathione (GSH) turnover in which oxidative stress affects the distribution of GSH between oxidized and reduced forms. Comparatively little is known of the turnover of other antioxidants.

Vitamin E (α-tocopherol, α-TH) is an important biological antioxidant and extensive research has been performed concerning its oxidation. Numerous oxidation products of α-TH have been described and further research is needed to determine the

roles and pathways in turnover for specific products. Most of the research done to model α-TH oxidation has been in biomimetic membrane preparations such as liposomes and microsomes. There is a need for further research in more complex cellular systems to further evaluate α-TH turnover during oxidative stress.

PAMs represent an ideal primary cell culture system with which to study α-TH oxidation. These cells are easily obtainable in large numbers and culture techniques are well described in the literature. PAMs have a variety of functions including phagocytosis, chemotaxis, cell recruitment and oxidant production. Activated macrophages are able to produce several radicals, including $O_2^{\cdot}$ and nitric oxide (NO), which in turn, are able to generate a variety of oxidants. The purpose of this research is to use cultured macrophages as a model system to study α-TH turnover.

FREE RADICALS AND OXIDATIVE INJURY

Free radicals are chemical species that have one or more unpaired electrons in the outermost electron shell. The stipulation that the unpaired electron(s) must exist in the outer most shell, excludes most transition metal ions from this classification (Halliwell and Gutteridge, 1990). However, many radicals are formed in and around living organisms including oxygen centered radicals, nitrogen centered radicals, carbon centered radicals, and sulphur centered radicals. Nitric oxide (NO) is synthesized by many cells. NO synthesis by phagocytes may contribute to bactericidal actions. Carbon centered radicals can occur from toxic exposures and by propagation from other radicals.

Exposure to CCl_4 leads to radical ($CCl_3\cdot$) formation by cytochrome P-450 metabolism, while lipid peroxidation is a good example of a propagation reaction. These radicals are transient in many free radical processes, but are rapidly converted to oxygen radicals in aerobic environments. Sulphur centered radicals also can be formed from cysteine sites on proteins and glutathione as the result of interaction with oxidants, often ending with the oxidation of thiols to disulfides and the formation of oxygen-centered radicals (McKay and Bond, 1985). Oxygen centered radicals, often responsible for the generation of carbon and sulphur radicals, are able to produce a variety of other oxidants and are available from a wide range of sources. Oxygen radicals predominate in biological systems because oxygen, a paramagnetic molecule, reacts readily with virtually all other cellular radicals. These qualities have made oxygen radicals a major focus of interest to many researchers.

Formation of Oxygen Radicals

Molecular oxygen is a diradical with two unpaired electrons in π^* antibonding orbitals. Oxygen is readily reduced to O_2^- as the result of a single electron reduction. In aqueous solutions, O_2^- behaves as a weak base and acts as a reducing agent. It is less reactive than in organic solution due to hydration, however it is acted upon by superoxide dismutase (SOD) enzymes that catalyze the dismutation reaction (1), which generates hydrogen peroxide (H_2O_2).

$$2O_2^-\cdot + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad (1)$$

H_2O_2 is not a radical compound and, like O_2^-, it is only moderately reactive and therefore cannot account for all the tissue damage associated with oxidative injury. In the presence of a metal ion catalyst, O_2^- and H_2O_2 can react to form the hydroxyl radical ($\cdot OH$) via the superoxide-driven Fenton reaction (2).

$$O_2^- + Fe^{3+} \longrightarrow Fe^{2+} + O_2$$

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

$$O_2^- + H_2O_2 \longrightarrow O_2 + \cdot OH + OH^- \qquad (2)$$

In this reaction, O_2^- acts as a reducing agent for ferric iron (Fe^{3+}) to produce molecular oxygen and ferrous iron (Fe^{2+}). Fe^{2+} can then undergo the Fenton reaction with H_2O_2 to form $\cdot OH$. The hydroxyl radical is extremely reactive and can interact with most biological molecules. It has the capability to induce the types of injury associated with oxidative stress.

There are a number of arguments made against the occurrence of superoxide-driven Fenton reactions *in vivo*. One concern is the presence of reducing agents other than O_2^- *in vivo*. Rowley and Halliwell (1982 a & b) studied the effects of different reducing agents on $\cdot OH$ production by the superoxide-driven Fenton reaction. These authors determined that NADH, NADPH or thiol compounds would not prevent O_2^- dependent generation of $\cdot OH$. However, the most prominent role for O_2^- appears to be the generation of H_2O_2. A second concern about this reaction was the availability of Fe^{3+}. *In vivo*, Fe^{3+} usually is sequestered within cells and the extracellular fluids by a

variety of proteins. Gutteridge and Halliwell (1987) measured iron ions in human body fluids with the bleomycin assay and found the availability of iron to be very low, often non-detectable. However, other researchers have found that oxidative stress can release iron for interaction with free radicals. Biemond *et al.* (1984) demonstrated that iron could be mobilized from ferritin by $O_2^{\cdot}$. Poppo and Halliwell (1988) found that H_2O_2 can degrade heme proteins to release free iron. Recently, evidence has been presented that suggests that $\cdot OH$ produced by H_2O_2 and copper ions caused characteristic damage to purine and pyrimidine bases of exposed DNA (Aruoma *et al.*, 1991). Through the formation of these different oxidants, a link has been established connecting the many different mechanisms of oxygen radical formation and oxidative injury.

Sources of Oxygen Radicals

Biological systems are continuously being assaulted by oxygen radicals derived from a wide assortment of sources. Components of air pollution, including volatile organic compounds, NO_x and SO_2, can all undergo photochemical reactions that generate oxygen radicals, including the very reactive $\cdot OH$ radical (Hippeli and Elstner, 1991). Ultraviolet radiation incident on the earth's surface, has been demonstrated to produce both H_2O_2 and $O_2^{\cdot}$ from irradiation of NADH and NADPH as well as other biomolecules (Cunningham *et al.*, 1985). Toxic exposures to environmental contaminants can also create radicals. Paraquat, a pulmonary toxicant, forms a paraquat radical cation in Type II cells. That radical can reduce molecular oxygen to $O_2^{\cdot}$ (Gutteridge and Toeg, 1982).

Other toxins have the ability to indirectly generate O_2^- by stimulating tissue macrophages. Asbestos causes fibrosis of the lung along with malignant cell transformations. Kamp *et al.* (1989) studied cell injury in cultured pulmonary epithelial cells and found that cytotoxic effects were governed by H_2O_2 release by asbestos-activated polymorphonuclear leukocytes. In addition to external sources of O_2^-, many biological enzyme systems are also able to manufacture these radicals.

All aerobic cells contain a group of enzymes known as cytochromes, which are heme-containing proteins involved in electron transport. The cytochrome P-450 enzymes are found in many tissues and these enzymes function by oxidizing potential toxins and exposing functional sites for conjugation and excretion. Releases of O_2^- in hepatocytes have been linked with cytochrome P-450 activity. Hildebrandt and Roots (1975) measured increases in H_2O_2 production in microsomes isolated from animals pretreated with phenobarbital which induced P-450 activity.

A more ubiquitous source of O_2^-, that can be found in all cell types, is the cytochrome-c oxidase of the respiratory electron transfer chain. Cytochrome-c oxidase is located in the inner membrane of the mitochondria and it functions to reduce molecular oxygen to water. However, a portion of the O_2 consumed is lost as O_2^- and H_2O_2. H_2O_2 in most cellular environments has a lifetime which permits diffusion over reasonable distances (Pryor, 1986) and it is able to cross lipid membranes that are impenetrable to O_2^-. These characteristics allow the leakage of H_2O_2 to be measured. Loschen *et al.* (1971) revealed that the rate of H_2O_2 production depended upon the metabolic state of

the mitochondria. The greatest rate took place in state 4 respiration, or in a state of ADP depletion, in which 1-2% of the total O_2 uptake generated H_2O_2 (Boveris and Chance, 1973). These data suggest that cellular respiration can produce oxidative stress, however cytochrome oxidases are not the only enzymes that generate oxidant species.

NADPH oxidase is an enzyme complex located in the plasma membranes of neutrophils, monocytes and tissue macrophages. These phagocytic cells are components of the immune system, whose primary function is to defend organisms against bacteria, parasites and tumor cells. In resting phagocytes, NADPH oxidase is inactive and dissociated into its components found in the plasma membrane, the cytoskeleton and the cytosol (Babior *et al.*, 1988). Upon stimulation of the phagocyte, the components of the oxidase are assembled in the plasma membrane and the enzyme becomes activated. NADPH oxidase reduces molecular oxygen to $O_2^{\cdot}$ by indirectly transferring an electron from NADPH. The binding site for NADPH is located on the cytosolic surface of the plasma membrane, while the oxygen binding site is exposed to the outer surface (Babior *et al.*, 1981). This configuration creates an electron flow out of the cell and generates $O_2^{\cdot}$ in the extra cellular matrix. Once in the extra cellular matrix, $O_2^{\cdot}$ is believed to participate in the superoxide-driven Fenton reaction which generates $\cdot OH$ as a toxic agent against foreign microbes and tumor cells.

In addition to $O_2^{\cdot}$, phagocytes, along with endothelial cells and neurons, are able to synthesize a second radical compound, NO. These enzymes catalyze the production of NO from the amino acid L-arginine through a N^G-hydroxy-L-arginine intermediate.

Two nitric oxide synthase enzymes have been identified; one is calcium-activated. Neurons and endothelial cells express this calmodulin- activated enzyme (Knowles *et al.*, 1989). The macrophage enzyme is not activated by calcium. Instead, Stuehr and Marletta (1987) demonstrated that freshly isolated macrophages need to be stimulated by either lipopolysaccharide (LPS) or gamma- interferon(IFN-γ) to elicit enzyme activity. The authors observed a lag phase of about 8 hours before synthesis of NO was observed. This lag phase is associated with protein synthesis for enzyme expression. Once synthesized, NO is not very reactive with biological molecules. However, it is theorized that NO and O_2^- can interact to form $\cdot OH$ (Beckman *et al.*, 1990). These NO and O_2^- can react rapidly to form the peroxynitrite anion ($ONOO^-$), which is stable enough anion at physiologic pH to diffuse to critical cellular molecules. When protonated $ONOO^-$ decays quickly and can form $\cdot OH$ (3).

$$O_2^- + NO \longrightarrow ONOO^- + H^+ \longleftrightarrow ONOOH \longrightarrow HO\cdot + NO_2\cdot \qquad (3)$$

The authors demonstrated that 25% of peroxynitrite forms $\cdot OH$ and $NO_2\cdot$ with the remainder forming nitrate (NO_3^-)(4).

$$NO_2\cdot + \cdot OH \longleftrightarrow NO_3^- + H^+ \qquad (4)$$

Mahoney (1970) reported that a 32% yield of $\cdot OH$ could be detected during a reaction of H_2O_2 and nitrite at pH 5. With physiological pH about 7.4, this study may appear irrelevant, however it has been reported that the actual pH beneath activated macrophages may be lower than 5 (Etherington *et al.*, 1981). These experiments suggest that NO may serve to enhance the bacteria killing activity of O_2^- by providing a second mechanism to

generate •OH. Thus, in performing their role in the immune response, phagocytes create a considerable oxidative stress for the surrounding tissues. When this stress is combined with those of respiration or chemical oxidants, the probability of interaction between radicals and biological molecules culminating in tissue injury significantly increases.

Molecular Mechanisms of Oxidative Injury

The tissue injury associated with exposure to oxygen free radicals involves the reactions between •OH and biological macromolecules. Because of their highly reactive nature, •OH radicals cause damage at the site of their formation. This site specific damage largely depends upon the location of catalytic metal ions. Therefore, a metal catalyst near a lipid membrane will most likely facilitate in lipid peroxidation, whereas a metal in association with DNA can cause strand breaks. DNA, proteins and lipids are all targets of oxidative attack by •OH. All these biological molecules are subject to hydroxylation and hydrogen abstraction reactions leading to radical species. In aerobic environments, these radical species quickly form peroxyl radicals which propagate and amplify damage through chain reactions. Many diverse and destructive reactions can occur in tissues exposed to oxidative stresses as illustrated in Figure 1. This scope of cell injury is best understood by considering individual types of oxidative damage to cellular lipids, DNA and proteins.

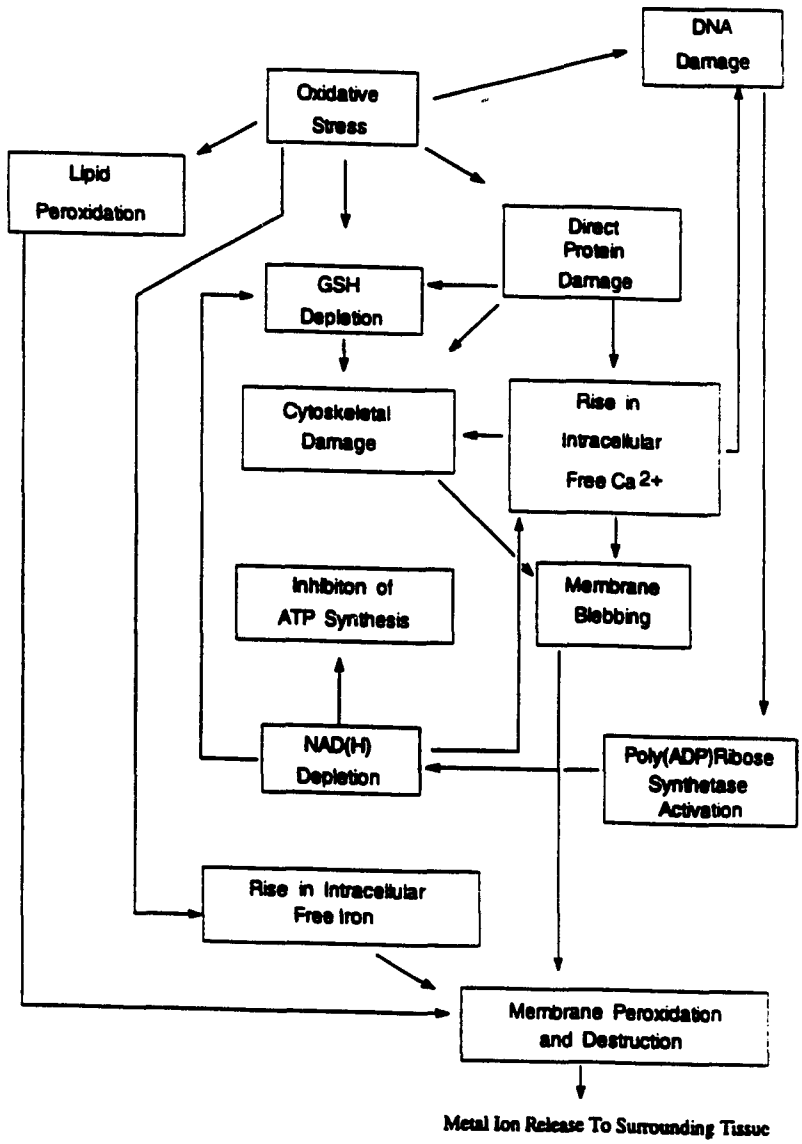


Figure 1. Interacting mechanisms of cell injury by oxidative stress (Halliwell and Gutteridge, 1992).

Lipid peroxidation, an important mechanism in all tissue injury caused by oxygen radicals, can be broken down into two steps, initiation and propagation. The initiation of peroxidation consists of a hydrogen abstraction from a polyunsaturated fatty acid by an initiating radical such as $\cdot OH$ (5).

$$R\text{-}CH_2\text{-}R' + \cdot OH \longrightarrow R\text{-}CH\cdot\text{-}R' + H_2O \qquad (5)$$

The carbon centered radical tends to rearrange to form a conjugated diene that can react in several ways. It can interact either with a second lipid radical to cross-link two fatty acids or with a membrane protein. In the presence of oxygen, the most prominent reaction for conjugated dienes is the generation of a peroxyl radical (6).

$$R\text{-}CH\cdot\text{-}R' + O_2 \longrightarrow R\text{-}CHO_2\cdot\text{-}R' \qquad (6)$$

The peroxyl radical can abstract a hydrogen from an adjacent lipid molecule to create a lipid hydroperoxide and a new carbon centered lipid radical (7).

$$R\text{-}CHO_2\cdot\text{-}R' + R''\text{-}CH_2\text{-}R^n \longrightarrow R\text{-}COOH\text{-}R' + R''\text{-}CH\cdot\text{-}R^n \quad (7)$$

This reaction is termed the propagation of lipid peroxidation. Lipid peroxidation can lead to increases in membrane permeability to ions provoking reductions in membrane potential (Beatrice *et al.*, 1980) and increases in intracellular Ca^{2+} compartmentation (Richter and Kass, 1991). Peroxidation can also decrease membrane fluidity and cause ruptures in cellular and organelle membranes. Fong *et al.* (1973) demonstrated that rat lysosomes can release their enzymes, as a result of membrane peroxidation, causing additional cellular damage from lysosomal enzymes. In addition to these cytotoxic events, some lipid peroxide fragments are themselves toxic. 4-Hydroxynonenal is a

cytotoxic aldehyde derived from a lipid cycloperoxide (Winkler *et al.*, 1984). This aldehyde reacts by binding with thiol groups on proteins and depletes glutathione (Cadenas *et al.*, 1983).

Oxygen free radicals can make changes in DNA directly by reacting with bases and deoxyribose, or indirectly by affecting enzyme activities. Direct reactions between •OH and DNA bases are typically identified either by an addition to the double bonds within these molecules or a hydrogen abstraction from the methyl group on thymine (von Sonntag, 1987). The addition reactions generate base radicals which lead to a variety of products that can be analyzed by GC/MS (Dizdaroglu, 1991). 8-Hydroxypurines and formamidopyrimidines are major products in mammalian chromatin exposed to H_2O_2 in the presence of ferric and cupric ions. Von Sonntag (1987) also described alterations in DNA sugar moieties as a result of hydrogen abstraction. These aberrations could lead to base release or strand breaks. Peroxyl radicals can also perform hydrogen abstractions to yield many of the same products. An additional type of •OH induced damage to DNA is cross linkage with proteins. Gajewski *et al.* (1988) studied calf thymus nucleohistone that had been exposed to ionizing radiation as a source of •OH. The authors reported thymine and cytosine products cross linked to a variety of amino acids analyzed by GC/MS. Other reactions involving proteins and •OH can have adverse affects on the DNA sequence by altering enzyme functions.

The oxidation of proteins by oxygen free radicals is characterized by alterations in amino acids, protein structure, hydrophobicity and susceptibility to proteolysis (Pacifici

and Davies, 1990). The first changes occur in amino acids by the formation of distinctive products such as bityrosine, a covalently bound biphenyl produced by dimerization of two tyrosine radicals (Prütz *et al.*, 1983). The authors suggest that these types of products are the result of hydrogen abstractions by ·OH. A second mechanism is the conversion of amino acids which is exemplified by the hydroxylation of phenylalanine to form tyrosine (Davies *et al.*, 1987). Finally, Levine (1983) suggested that carbonyl formation may also serve as an early indicator of protein oxidation. As the oxidation of amino acids progresses, the secondary and tertiary structures change as well. In aerobic conditions, peroxyl radical attack on proteins can yield many of the same products. These changes alter overall isoelectric points, folding and hydrophobicity of proteins (Pacifici and Davies, 1990), modifying enzyme specificity and activity. Protein aggregates form as a result of intermolecular covalent cross-linking and the appearance of hydrophobic patches on proteins (Davies and Delsignore, 1987). As a result, enzymes involved with DNA repair and replication can be functionally modified or inactivated by oxygen free radicals. All these observations demonstrate the mutagenic and carcinogenic potential of oxygen free radicals. Therefore, oxygen free radicals have been determined to be strong tumor promoters and implicated in the mechanisms of other promoters such as TPA and UV radiation. In addition to cancer, there are other disease states associated with oxygen radicals and their interactions with both proteins and lipids.

Symptoms and injuries associated with a number of diverse diseases including atherosclerosis, adult respiratory distress syndrome (ARDS) and rheumatoid arthritis, are

postulated to include mechanisms involving the recruitment of phagocytes and generation oxygen radicals. The juxtaposition of lipid and protein oxidation occurs in lipoproteins, whose oxidation leads to atherosclerosis. Radicals are theorized to peroxidize low-density lipoproteins (LDL). The LDL-peroxide is internalized by macrophages within the blood vessel forming foam cells (Haberland and Fogelman, 1987) that make up atherosclerotic plaques. Experiments by Carew *et al.* (1987) examined the effects of chain-breaking anti-oxidants on atherosclerosis in rabbits. They found that rabbits treated with the anti-oxidant probucol had a significantly lower plaque accumulation. These results are consistent with the theory of LDL peroxidation.

Depletion of glutathione (GSH), along with other antioxidants, is a reported symptom with many diseases associated with radical injury. Patients with adult respiratory distress syndrome (ARDS) have demonstrated significantly lower level of glutathione in their alveolar fluid (Pacht *et al.*, 1991). The authors interpret the lower levels to be a consequence of decreased secretion of GSH into the alveolar fluid by lung cells due to oxidative stress. Highly oxygenated tissues, such as the CNS, lungs and heart, are under extreme oxidative stress and they are in need of protection. Oxygen radicals have been demonstrated to be present in large numbers from an ample variety of sources and intensely damaging in their interactions with biological molecules. Biological systems have evolved in the presence of this oxidative threat and they have countered it with antioxidants.

ANTIOXIDANTS

Antioxidants can be loosely defined as a group of compounds that protect tissues from oxidative injury. As previously discussed, oxygen free radicals from different origins induce injury by reacting with DNA, proteins and lipids. Antioxidants consist of a diverse assortment of molecules that protect tissues by specific mechanisms. These molecules consist of antioxidant enzymes, simple peptides, protein complexes, and naturally occurring carotenoids and tocopherols. Antioxidants can be grouped into three categories based upon their mechanisms of action. The first category consists of antioxidants that inhibit radical formation by sequestering metal catalysts. The second group scavenges precursors to reactive radicals. This can be accomplished by dismutating superoxide anions or enzymatically depleting H_2O_2. A third category of antioxidants consists of those that trap radicals and terminate propagation reactions. In addition to their antioxidant functions, these compounds can participate in other roles such as xenobiotic detoxication, antioxidant regeneration and substrate transportation.

Metal Binding Proteins

Organisms use proteins to transport and store transition metals to limit the amount of "free" metal ions available for generation of oxygen radicals. Two-thirds of the body's iron is bound to hemoglobin, with smaller amounts in myoglobin, various enzymes and the transport protein transferrin (Halliwell and Gutteridge, 1990). Transferrin is a glycoprotein found mainly in the plasma, it acts as an antioxidant by

binding Fe(III) at physiologic pH. Plasma transferrin is only 30% loaded under normal conditions and the resulting level of free iron in plasma has been demonstrated to be non-detectable (Hershko *et al.*, 1978). Lactoferrin, a protein similar to transferrin, is found in several body fluids such as milk and saliva. It is also produced by phagocytic cells, where its antioxidant capacity is theorized to protect surrounding tissue from phagocytic radical production (Halliwell *et al.*, 1985). Iron, when not associated with any of these other proteins, is stored in ferritin. Ferritin acts as a protein coat that inhibits interaction with any oxygen species. Iron-binding proteins serve to protect tissues from the participation of iron in the Fenton reaction. However, other transition metals, such as copper, are also capable of generating $\cdot OH$. About 95% of plasma copper is bound to ceruloplasmin. Ceruloplasmin is a protein that functions in a similar fashion to transferrin and ferritin. It has additional antioxidant capabilities by catalyzing the oxidation of Fe^{2+} to Fe^{3+} (Osaki *et al.*, 1989).

Antioxidant Enzymes

Superoxide dismutase catalyzes the dismutation of O_2^- to H_2O_2 by reaction (1). SOD is a widely distributed copper and zinc containing enzyme that exists in a variety of forms (Fridovich and Handler, 1961). SOD activity is found in the cytosol (Cu-Zn enzyme) and in other subcellular compartments, such as mitochondria (Mn enzyme) associated with O_2^- production (Chance *et al.*, 1979). Its activity increases linearly with the increase in concentration of O_2^- (Rotilio *et al.*, 1972). The mechanism of action of

the Cu-Zn enzyme uses the successive reduction (8) and oxidation (9) of copper to oxidize O_2^- (Fielden *et al.*, 1974).

$$SOD\text{-}Cu^{2+} + O_2^- \longrightarrow SOD\text{-}Cu^{+} + O_2 \qquad (8)$$

$$SOD\text{-}Cu^{+} + O_2^- + 2H^+ \longrightarrow SOD\text{-}Cu^{2+} + H_2O_2 \qquad (9)$$

This mechanism resembles those of copper ions and Cu^{2+}-amino acid complexes (Joester *et al.*, 1972; Rabani *et al.*, 1973). However, SOD has surface charges that orient O_2^- in a manner to facilitate the dismutation reaction by four orders of magnitude above the uncatalyzed rate (Getzoff *et al.*, 1983).

SOD supplies a necessary antioxidant function when working in conjunction with other H_2O_2-removing enzymes. One such enzyme is catalase, which is highly specific for hydrogen peroxide, but also metabolizes methyl and ethyl hydroperoxides. It catalyzes the dissociation of H_2O_2 to H_2O and O_2 by reactions 10 and 11 (Chance *et al.*, 1979).

$$\text{catalase} + H_2O_2 \longrightarrow \text{compound I} \qquad (10)$$

$$\text{compound I} + H_2O_2 \longrightarrow \text{catalase} + 2H_2O + O_2 \qquad (11)$$

Catalase contains a ferric heme iron and compound I is a short-lived ferric peroxide, where two electrons are transferred to H_2O_2 from Fe^{3+}. Chance *et al.* (1979) reviewed catalase activity and stated that enzyme activity varies directly with H_2O_2 concentration. As a result, catalase can control intracellular levels of H_2O_2 over a wide range of SOD activities.

The clearance of H_2O_2 and other peroxides is also performed by the glutathione

redox cycle. Reduced glutathione (GSH) is a ubiquitous tripeptide that directly or indirectly functions in a multitude of cellular processes. It is the most prominent nonprotein cell thiol; therefore it supplies an excellent source of reducing equivalents for oxidant interaction. The antioxidant role of GSH is mediated largely by the GSH redox cycle (Figure 2). GSH can be oxidized to form a disulfide, GSSG, by a number of mechanisms. Once generated, GSSG can be reduced back to GSH by reaction with glutathione reductase and NADPH. GSH can act as an antioxidant either by depleting H_2O_2 or by directly interacting with radical species. Glutathione peroxidase catalyzes reactions of GSH with hydroperoxides (12).

$$ROOH + 2GSH \longrightarrow ROH + GSSG + H_2O \qquad (12)$$

This enzyme is specific for GSH but, unlike catalase, it can react with many types of hydroperoxides, including phospholipidhydroperoxide. GSH peroxidase is a cytosolic enzyme with a significant amount also appearing in the mitochondria (Flohé, 1974). The enzyme has four selenium atoms which are theorized to be part of the reaction center (Flohé *et al.*, 1973). Wendel *et al.* (1975) demonstrated using X-ray photoelectron spectroscopy that a selenium-substrate moiety undergoes redox changes.

GSH can also act as a radical trapping agent by the abstraction of the cysteine hydrogen (13). The ensuing glutathione radical can form a disulfide bond with a second radical producing GSSG (14).

$$GSH + R\cdot \longrightarrow GS\cdot + RH \qquad (13)$$

$$GS\cdot + GS\cdot \longrightarrow GSSG \qquad (14)$$

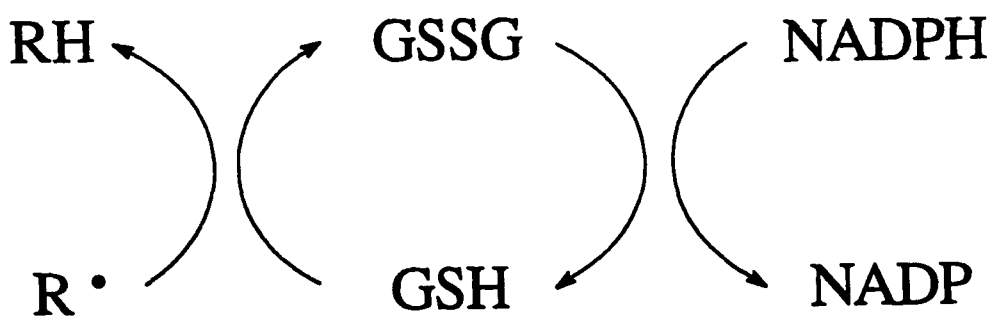


Figure 2. The overall summary of the glutathione redox cycle, the conversion of GSH to GSSG is also mediated by H_2O_2 (peroxidase) and disulfides (transhydrogenase).

Because of its diverse reactions and its ability to be recycled, GSH represents an important antioxidant system protecting tissues from constant assault by oxygen radicals.

Chain-breaking Antioxidants

Organisms make use of a number of naturally occurring antioxidant molecules. Generally, these molecules function by scavenging radical compounds. In a similar function, GSH has a hydrogen abstracted by a radical, then forms a disulfide bond to stabilize its radical species. Other antioxidants scavenge radicals by different mechanisms. One such group of molecules are the carotenoids, of which ß-carotene is the most widely studied. Recently, ß-carotene has been the focus of much attention due to its apparent anticarcinogenic effects. It is believed these effects are by virtue of its antioxidant capability (Pryor, 1991). It has been long known that ß-carotene is an effective singlet oxygen quencher. The radical scavenging reactions of ß-carotene are less understood. Burton and Ingold (1984) demonstrated that ß-carotene, in chlorobenzene under low oxygen tension, could prevent lipid peroxidation. In contrast, no antioxidant effect was seen in aqueous micelles when presented with an oxidative stress under 100% oxygen atmosphere (Pryor *et al.*, 1988). Krinsky and Deneke (1982) suggested that carotenoids could behave as antioxidants by acting as a competitive substrate for oxidative reactions. Handelman *et al.* (1991) addressed this issue by comparing oxidation products of ß-carotene in toluene under spontaneous autoxidation and found no difference in product formation between autoxidation and radical induced

oxidation. The authors hypothesize the formation of a carotenoid peroxy radical that breaks down to form the apo-carotenal series products. Kennedy and Liebler (1992) demonstrated that the antioxidant efficiency varies indirectly with pO_2. These authors found that the radical scavenging by ß-carotene is most effective at physiologic pO_2. Further investigation will be required to elicit an antioxidant mechanism for ß-carotene. There are, although, other compounds which have more clearly defined mechanisms of action.

Vitamin C, ascorbic acid (AH_2), is involved in a wide range of cellular processes (Moser and Bendich, 1991). It can act as an antioxidant by directly scavenging radical molecules. At physiologic pH, vitamin C largely exists as the ascorbate anion (AH^-) that can be reversibly oxidized to form the ascorbyl radical ($A^-\cdot$) (Laroff *et al.*, 1972). This radical is reasonably stable due to delocalization of the unpaired electron. The ascorbyl radical can either be further oxidized by other radicals (15), or undergo disproportionation (16) (Bielski, 1982).

$$A^-\cdot + R\cdot + H^+ \longrightarrow A + RH \qquad (15)$$

$$2A^-\cdot + H^+ \longrightarrow AH^- + A \qquad (16)$$

In both reactions, an ascorbyl radical is lost to dehydroascorbate, which can be reduced to ascorbate by GSH or thiols. Ascorbate is able to act as a prooxidant by generating O_2^- through its autoxidation in the presence of transition metals. The antioxidant efficiency of ascorbate decreases as the concentration of ascorbate increases because of increased competition of the autoxidation reaction (Bendich, 1986). Although ascorbate

has been demonstrated to trap peroxyl radicals in aqueous medium, it may not react efficiently with lipid peroxyl radicals in membranes. In the presence of vitamin E (α-TH), however, ascorbate does demonstrate increased antioxidant protection of liposomes (Scarpa *et al.*, 1984). This protection is thought to be due to the regeneration of the α-tocopheroxyl radical (α-T•) by ascorbate (17-18).

$$\alpha\text{-TH} + \text{ROO}\cdot \longrightarrow \text{ROOH} + \alpha\text{-T}\cdot \qquad (17)$$

$$\alpha\text{-T}\cdot + \text{AH}^- \longrightarrow \alpha\text{-TH} + \text{A}^-\cdot \qquad (18)$$

This reaction helps maintain the level of vitamin E, the most important lipid soluble molecule in the defense against lipid peroxidation.

VITAMIN E

Vitamin E consists of a family of sterically hindered phenolic antioxidants, the tocopherols. The tocopherol molecules are composed of two portions, a chromanol ring and a phytyl carbon chain. There are several tocopherol molecules, α, γ, and δ that differ only in the number and location of methyl groups on chromanol ring. α-TH has three methyl groups on the 5, 7 and 8 carbons and is the most abundant tocopherol in animal tissues (Figure 3). Of the eight possible stereoisomers, only the 2*R*,4'*R*,8'*R* isomer of α-TH occurs naturally in most foods (Sokol, 1988). The chromanol ring structure is responsible for the antioxidant capabilities of α-TH, whereas the phytyl chain anchors α-TH in the lipid bilayer.

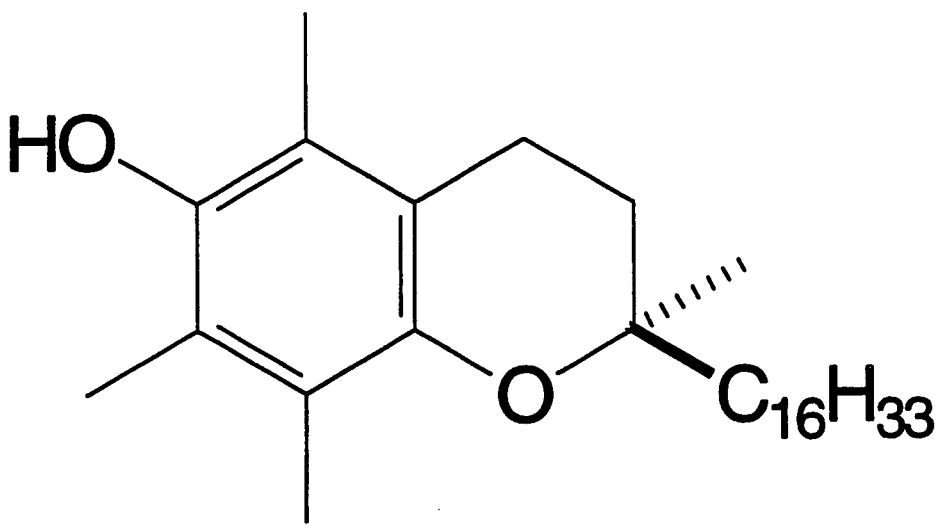


Figure 3. 2*R*, 4'*R*, 8'*R*-α-Tocopherol (α-TH)

Distribution and Transport

All the tocopherols occur naturally and are available in most diets. Of these, α-TH has the greatest activity and is present in the largest amounts *in vivo*. Behrens and Madere (1987) investigated the relative absorption, transport and retention of α-TH and γ-TH. They found that γ-TH uptake and retention was inversely proportional to the α-TH level in all tissues sampled. The authors conclude that absorption and transport of vitamin E is a saturatible process with a much higher affinity for α-TH. α-TH is absorbed intestinally in a lipid-bile micelle along with free fatty acids, monoglycerides and other fat-soluble compounds. Once in the enterocyte, vitamin E is incorporated into chylomicrons and transported to the liver by the mesenteric lymphatics. In the liver, chylomicrons are hydrolyzed and the free fatty acids are reesterified to form triglycerides. Vitamin E is assembled with these glycerides into very low density lipoproteins (VLDL). The liver secretes vitamin E into the plasma within these VLDL. Experimental results have demonstrated that the liver exhibits a strong preference for the incorporation of α-TH over other tocopherols (Traber and Kayden, 1989). VLDLs are catabolized to low density lipoproteins (LDL) and high density lipoproteins (HDL). Most systemic transport of α-TH appears to be accomplished by LDL and HDL. Mechanisms for the distribution of α-TH within tissues and cells are in need of further research.

Evidence has been presented that documents exchange of α-TH between lipoproteins and erythrocytes (Bjornson *et al.*, 1975). Granot *et al.* (1988) reported that this exchange of α-TH does not require a transfer protein. Other tissues may receive

α-TH from cells expressing LDL receptors on their surfaces such as PAM (Brown and Goldstein, 1986). Additional investigations demonstrated that cellular α-TH increases following incubation with LDL (Traber and Kayden, 1984). This increase was only seen in cells that expressed LDL receptors. *In vitro* studies have implicated lipoprotein lipase in a mechanism of α-TH uptake (Traber *et al.*, 1985). This enzyme catalyzes the lipolysis of triglyceride rich lipoproteins and may act as a transfer protein for α-TH. Tissues that express lipoprotein lipase activity, like the brain, muscle and adipose tissue, may receive a significant amount of α-TH in this manner (Burton and Traber, 1990).

After being taken up by individual cells, α-TH is most concentrated in the lipid bilayer of membrane-rich organelles, such as mitochondria, microsomes and nuclear membrane (Machlin, 1990). Cytosolic proteins have been implicated with intermembrane exchange of α-TH between organelles (Mowri *et al.*, 1981; Murphy *et al.*, 1981). Once in its target membrane, α-TH is not believed to be cemented in place (Burton and Ingold, 1986). The phytyl tail acts to secure α-TH within the membrane, but the molecule can maneuver laterally around the membrane. As a result, it is not uniformly distributed within the membrane. Kagan *et al.* (1990) reported that α-TH is found in higher concentrations around domains rich in polyenic phospholipids. With the ability to move between and within cellular membranes, α-TH may diffuse to areas in need of its antioxidant capabilities.

Antioxidant Chemistry of Vitamin E

α-TH is one of the most efficient antioxidants available (Burton *et al.*, 1985). These authors reported that α-TH reacts with peroxyl radicals 200 times faster than the commercially available antioxidant, butylated hydroxytoluene (BHT). α-TH derives its antioxidant function from the structure of its chromanol ring. The phenolic hydrogen can be abstracted by a radical species to generate a stable product and the tocopheroxyl radical α-T• (19).

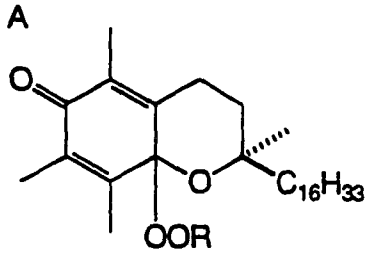
$$R\bullet + \alpha\text{-TH} \longrightarrow RH + \alpha\text{-T}\bullet \qquad (19)$$

The α-T• radical is stabilized by the chromanol ring, which disperses the unpaired electron density over its entire structure. This radical is unable to abstract hydrogen from surrounding biomolecules, therefore α-TH makes an excellent radical trapping compound. Because of its location within the lipid bilayer, α-TH is the primary defense against lipid peroxidation. The rate constant for the hydrogen abstraction from α-TH is two to three orders of magnitude greater than that for lipid peroxyl radical propagation (Howard and Ingold, 1967). As a result, α-TH offers antioxidant protection at the average concentration of one molecule for every one thousand phospholipid molecules (Burton *et al.*, 1983). The α-T• can undergo both oxidation and reduction reactions. Ascorbate is believed to be able to reduce α-T• back to α-TH by donating an electron as in equation (18). Packer *et al.* (1979) demonstrated the ascorbate-dependent formation of α-TH from α-T• in isopropanol:acetone:water solution (5:1:4). The authors reported the reduction of the radicals proceeded with a rate constant of 1.55×10^6 $M^{-1} \cdot s^{-1}$.

Further evidence for this recycling reaction was provided by Scarpa *et al.* (1984). These authors reported a rate constant of 2 X 10^5 $M^{-1} \cdot s^{-1}$ for the same reaction in a phosphatidylcholine liposome system. This sparing of α-TH by ascorbate has also been implicated *in vivo* (Bendich *et al.*, 1984). These studies observed α-TH levels in guinea pigs fed diets with varying amounts of vitamins C and E. The lungs and plasma of subjects with vitamin C enhanced diets had significantly higher levels of α-TH than those on a lower vitamin C diet. The interaction between α-TH and ascorbate may link aqueous antioxidants with lipid peroxyl radical trapping and greatly enhance the ability of biological systems to deal with oxidative stress in lipid membranes.

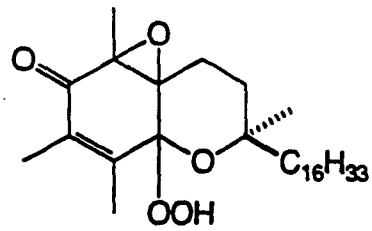
If α-T• is not reduced to α-TH, it is available for oxidation reaction with a second radical. These reactions can yield a variety of products. Winterle *et al.* (1984) described the formation of 8a-(alkyldioxy)tocopherones (Figure 4A) after exposing α-TH to azo*bis*-(2,4-dimethylvaleronitrile) (AMVN). AMVN generates peroxyl radicals and causes the oxidation of α-T• by an addition reaction at the 8a carbon of the chromanol ring. Additional products were isolated by Yamauchi *et al.* (1989). These authors reported the presence of a pair of epoxy-(8a-ethoxy) tocopherones when α-TH was exposed to AMVN in ethanol. These products are believe to be the result of solvent reaction with a pair of epoxy-8a-hydroperoxytocopherones (Figure 4B & 4C). The epxoy-tocopherones appear to be generated in a reaction competing with the 8a-tocopherone (Matsuo *et al.*, 1989; Liebler *et al.*, 1990). In this reaction, a peroxyl radical epoxidizes α-T•. This epoxy- α-T• then adds an oxygen molecule at the 8a

A



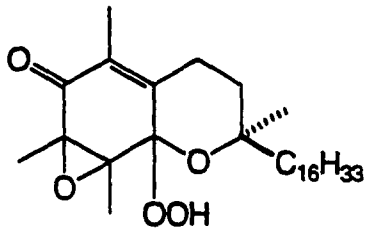
8a-(alkyldioxy)tocopherone

B



epoxy-8a-hydroperoxytocophreone

C

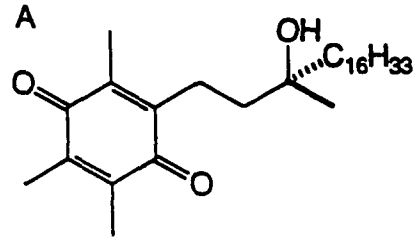


epoxy-8a-hydroperoxytocopherone

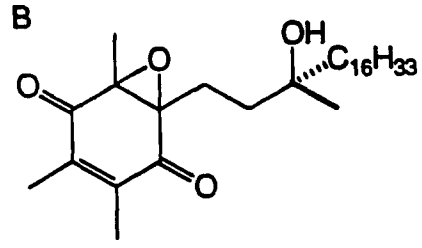
Figure 4. **The structures of 8a-(aklyldioxy)tocopherones and epoxy-8a-hydroperoxytocopherones.**

position and abstracts a hydrogen to form the epoxytocopherone. The epoxytocopherones and the 8a-alkyldioxytocopherones are the primary products of tocopheroxyl radical oxidation. In membrane models during metal catalyzed lipid peroxidation, the electron transfer reactions generate 8a-hydroxytocopherones and the epoxytocopherones rather than 8a-(alkyldioxy)tocopherones (Liebler *et al.*, 1992). When these tocopherones are formed in the presence of water, hydrolysis of these products rapidly occurs. This hydrolysis of the epoxytocopherones results in the formation of the respective α-tocopherolquinone-2,3-oxide (TQE1) and α-tocopherolquinone-5,6-oxide (TQE2) (Figure 5B & C), while the hydrolysis of the 8a-(alkyldioxy)tocopherones yields α-tocopherolquinone (α-TQ) (Figure 5A). All three of these products are considered irreversibly oxidized and are thought not to be reduced back to α-TH in biological systems. Because α-TQ and the TQEs are "dead end" products of α-TH oxidation, they may be useful indicators of α-TH consumption and thus of oxidative stress.

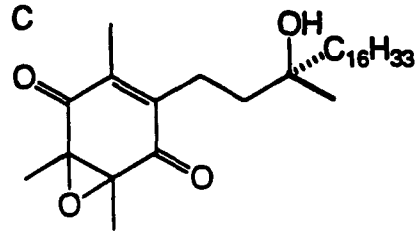
Recently, there has been a large interest in developing assays for oxidative stress. Lipid peroxidation has long been known as a result of oxidative stress. Many researchers measure products of lipid peroxidation as indicators of oxidative stress. All these assays are very effective at measuring damage to biological molecules as a result of oxidative stress. However, measuring the amount of oxidative damage to a biological tissue may not be an accurate evaluation of the oxidative stress which that tissue is under. Antioxidants protect tissues against oxidative injury before it can occur and much of the injury that does occur is repaired.



alpha-tocopherolquinone



alpha-tocopherolquinone-2,3-oxide



alpha-tocopherolquinone-5,6-oxide

Figure 5. The structures of α-tocopherolquinone and the α-tocopherolquinone epoxides.

There is evidence that antioxidants are depleted to some extent before oxidative damage occurs in biological systems. Therefore, tissues may be under oxidative stress before any sign of damage to biological molecules. An alternative approach to measurement of oxidative stress is to measure the state of antioxidants. The use of the glutathione redox cycle has been offered as a possible measure of oxidative stress. GSH reacts with hydroperoxides and glutathione reductase to form GSSG and other products (reaction 12). However, the level of GSSG is not a good measure of oxidative stress because GSSG is readily reduced. Vitamin E turnover may represent a better indicator of oxidative stress.

The phrase "vitamin E turnover" is here defined as the loss of the antioxidant molecule, α-TH, from the biological system. Vitamin E turnover in tissues may encompass three processes, oxidation, physical exchange and possibly metabolism. The metabolism of vitamin E is not well understood. There is presently little basis for distinguishing metabolic and antioxidant reactions of vitamin E. It is reasonable to believe that common enzymes, such as epoxide hydrolase, may react with vitamin E oxidation products. The oxidation of vitamin E has been reasonably well characterized in model systems. Figure 6 represents the possible reactions of α-TH. This figure demonstrates the variety of products that α-TH oxidation can produce. As seen previously, α-TH can have a hydrogen abstracted and form the relatively stable α-T$\cdot$ (Figure 6, reaction 1). The α-T$\cdot$ can undergo reactions with either ascorbate (Figure 6, reaction 2) or with a second peroxyl radical (Figure 6, reaction 3). These reactions

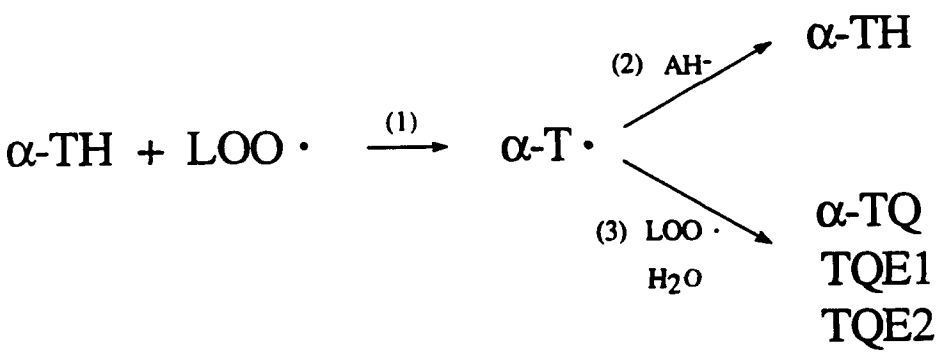


Figure 6. A schematic representation of the competing reactions for α-tocopheroxyl radicals (α-T·). Reaction (1) is the hydrogen abstraction by a lipid peroxyl radical (LOO·). The resulting α-T· can either be reduced by ascorbate (AH^-·), or oxidized by a second lipid peroxyl radical.

will regenerate α-TH or produce α-TQ and the TQEs, respectively. In the case of low levels of oxidative stress, the concentration of peroxyl radicals will also be low. Therefore, the chance of a second peroxyl radical interacting with α-T• before being reduced back to α-TH is lower. As the concentration of peroxyl radicals increase, so should the rate of production of α-TQ and the TQEs. The final component of turnover is physical loss of α-TH. Oxidative stress may be reflected by changes in how tissues exchange unoxidized α-TH. These last two components are especially important until more information can be acquired concerning metabolism.

Vitamin E oxidation has been studied in a number of model systems. Numerous studies have described α-TH oxidation in homogeneous solutions by various radical generating compounds. These systems permit the oxidation to be studied under chemically defined conditions. Several studies of the α-TH peroxyl radicals in solution have been reported (Matsuo *et al.*, 1989; Yamauchi *et al.*, 1989; Liebler *et al.*, 1990). Liebler *et al.* (1990) studied the oxidation of α-TH by peroxyl radicals generated by the thermolysis of AMVN in acetonitrile and acetonitrile/water mixtures. In the presence of water, the tocopherone and epoxytocopherones were hydrolyzed to α-TQ (5A) and TQEs(5B, 5C), respectively.

α-TH oxidation during iron-catalyzed liposome peroxidation yielded epoxytocopherones (4B, 4C), epoxy quinones (5B, 5C), and α-TQ (5A) (Liebler *et al.*, 1992). However, α-TQ was not formed from the 8a-(alkyldioxy)tocopherones, which were not detected as products. Instead, α-TH and α-T• react with radicals by electron-

transfer to yield 8a-hydroxytocopherone, which rearranged to α-TQ. These experiments demonstrate that competition for α-T• in complex systems can have an effect upon product distribution.

To determine the effect of membrane composition on α-TH antioxidant functions, Mehlhorn *et al.* (1989) compared α-TH levels in liposomes, microsomes and mitochondria. These authors found similar α-TH decay rates among the different membrane preparations. As result, there may also be similar oxidation pathways in different membranes regardless of their composition. Buttris and Diplock (1988) determined that α-TH preferentially distributed to membranes with high degrees of unsaturation. So, although the oxidation kinetics appear to be unaffected by the complexity of membranes, composition may affect α-TH oxidation by its distribution.

To determine the behavior of α-TH in more complex systems, whole cell models have also been employed. The relationships between α-TH, GSH and Ca^{2+} efflux has been extensively studied in hepatocytes (Pascoe *et al.*, 1987; Fariss *et al.*, 1985). Treatment with α-TH inhibited the depletion of GSH and increases of cytosolic Ca^{2+} seen in oxidatively challenged hepatocytes. Glascott *et al.* (1992) also studied hepatocytes to determine the effect of α-TH on oxidative cell death. These experiments demonstrated *in vitro* that α-TH can effectively prevent oxidative cell death. They also reported that cultured hepatocytes secreted α-TH into the culture medium. Other cell systems have been used to examine the effect of α-TH on a variety of parameters. Sharmanov *et al.* (1990) used peritoneal macrophages to study the mutually supportive relationship between

vitamin E and other antioxidant enzymes. Peritoneal macrophages were used to determine that there is an inverse relationship between α-TH concentration and both PGE_2 and O_2^- production (Sakamoto *et al.*, 1991). Coquette *et al.* (1986) used them to look at the effect of macrophage activation on α-TH levels. These authors found that activation led to a 40% decrease in α-TH in one hour. Little information, however, is presently available about α-TH turnover in cell culture. Additional study of the contributions of oxidation and physical exchange to α-TH turnover in cells is needed. Once determined, these relationships will allow the association between oxidative stress and α-TH turnover to be better defined. A cell system for such studies should display susceptibility to oxidative stress, be readily isolated in reasonable quantity, contain measurable amounts of α-TH and maintain viability in primary culture.

PULMONARY ALVEOLAR MACROPHAGES

PAM are the resident phagocytic cells of the lungs. These cells are involved in the pulmonary immune response to pathologic organisms and substances. PAM have a large diversity of receptors which allow them to respond to a wide variety of stimuli. Once stimulated, PAM release numerous secretory products that mediate their many effects. PAM are involved in promoting the inflammation response through the release of cytokines and arachidonic acid metabolites. They modulate inflammation by the release of protein inhibitors. As part of this inflammation response, PAM can recruit other PAM and neutrophils by the release of chemotactic factors. Once recruited, all

these cells have the ability to respond with a "respiratory burst". The respiratory burst describes the increase in oxygen consumption resulting from the production of $O_2^{\cdot}$. The radicals and oxidants generated by PAM and neutrophils are a component of their bactericidal activities. In addition, PAM are involved in phagocytizing particles, debris and organisms. These functions maintain a clean and sterile environment in the bronchioles and alveoli.

PAM have been demonstrated in both human and animal models to be derived from peripheral blood monocytes (van Oud Ablas and van Furth, 1979). Although some investigators have reported replication of PAM in the lung, the primary source appears to be monocyte infiltration (Fels and Cohn, 1986). Subpopulations of PAM have been described based upon heterogeneity of receptor expression and PAM functions (Chandler *et al.*, 1986). Phagocytic cells have been determined to possess at least 50 different receptors on the surface of their plasma membranes (Hamilton and Adams, 1987). PAM contain a large variety of Fc receptors that bind several immunoglobulins and complement fragments, and these receptors appear to govern phagocytic functions (Reynolds *et al.*, 1975). In addition to Fc receptors, there are numerous receptors present on PAM for cytokines such as interleukin-2, tumor necrosis factor, and leukotriene. These receptors all appear to induce macrophage activation (Sibille and Reynolds, 1990). Other receptor types appear on the surface of PAM that mediate different activities. For example, glucocorticoid receptors mediate a down regulation of macrophage activity (Werb *et al.*, 1978). These receptors acting individually or

collectively are able to stimulate any combination of PAM responses.

The stimulation of macrophages to perform their assorted immune functions is termed *activation*. PAM activation is known to occur through two pathways of signal transduction as the result of ligands binding to their respective receptors (Berridge, 1985). The first pathway consists of the activation of a membrane G-protein that in turn activates adenylate cyclase. The activation of adenylate cyclase increases intracellular concentrations of cyclic-AMP. Cyclic-AMP is a powerful activator of protein kinase A, which leads to the phosphorylation of a number of proteins. This phosphorylation appears crucial to PAM activation. The second pathway consists of a G-protein-dependent activation of phospholipase-c which converts phosphatidylinositol 4,5-biphosphate to inositol triphosphate and diacylglycerol. Inositol triphosphate is implicated with regulation of cytosolic calcium, which, in turn, also plays a role in protein phosphorylation. Phorbol esters such as PMA appear to be able to mimic this pathway for PAM activation (DeChatelet *et al.*, 1976).

Activated PAM function by releasing secretory products. PAM release a wide range of products including enzymes, cytokines, bioactive lipids and oxidants. Macrophages express a variety of enzyme activities such as glycosaminidase, elastases, collagenases, acid hydrolases and various converting enzymes. This diverse enzyme release allows PAM to function in bacterial killing, complement activation and matrix renovation (Sibille and Reynolds, 1990). PAM regulate their enzymatic activity by the release of antiproteases like alpha$_2$-M (White *et al.*, 1980). The bactericidal function of

PAM is enhanced by their release of oxygen radicals.

PAMs are able to enzymatically generate two types of radicals, O_2^- and NO. O_2^- is primarily generated by a plasma membrane bound enzyme, NADPH oxidase. Babior *et al.* (1981) characterized the NADPH oxidase as an enzyme with two components. The first component binds NADPH on the cytoplasmic surface of the plasma membrane. The O_2^- formation takes place at a site on the enzyme located within the plasma membrane. The oxidation of NADPH makes an electron available for the reduction of molecular oxygen which is formed within or on the outer surface of the plasma membrane (Fantone and Ward, 1982). Once generated, O_2^- can react with SOD to form H_2O_2 (reaction 1). In the presence of a metal catalyst, O_2^- and H_2O_2 can react to form $\cdot OH$ (reaction 2). Activated neutrophils are believed to release lactoferrin, which can interact with lysosomal components to yield an iron catalyst (Ambruso and Johnston, 1981). Therefore PAM activation of NADPH oxidase can lead to the production of O_2^-, H_2O_2 and $\cdot OH$.

Additional reactive oxygen species can be produced by myeloperoxidase (MPO). This is an enzyme found in neutrophils and monocytes. PAM can acquire MPO enzymes by the phagocytosis of neutrophils and then incorporate them into their own membranes (Leung and Goren, 1989). This enzyme reacts with H_2O_2 and Cl^- to form hypochlorous acid (HOCl). HOCl is believed to participate in bacterial killing by the halogenation and oxidation of surface molecules of foreign organisms (McRipley and Sbarra, 1967). The decarboxylation of amino acids is also believed to play a role in HOCl cytotoxicity

(Strauss *et al.*, 1970). All the reactive oxygen species present stresses to foreign organisms, however oxygen radicals are not the only radicals produced by macrophages.

Macrophages have been demonstrated to contain a nitric oxide synthase enzyme which generates NO from L-arginine (Iyengar *et al.*, 1987). The enzyme isolated from murine macrophage uses NADPH and does not require Ca^{2+} (Hevel *et al.*, 1991). NO production by macrophages appears to depend upon specific sequence of stimulation. Where as superoxide production can be immediately stimulated by treatment with PMA, NO synthesis requires pretreatment with LPS (Lorsbach and Russell, 1992). As shown in reaction 3 (see above), NO can form $ONOO^-$ by interacting with $O_2^{\cdot-}$. The protonated form, HOONO, forms $\cdot OH$ and $NO_2\cdot$. $NO_2\cdot$ is highly toxic and can initiate lipid peroxidation and nitrosylate aromatic amino acids (Prütz *et al.*, 1985).

Cultured PAMs have the ability to generate an oxidative stress without the addition of a radical generating system. This ability makes cultured PAMs an extremely attractive cell model to study the *in vitro* turnover of α-TH. *In vitro* characteristics of PAMs have been extensively characterized and culture conditions favorable to many PAM functions are well described (Adams, 1979). These cells are obtained relatively easily from a variety of animals by broncho-alveolar lavage in sufficient numbers to perform analytical procedures. PAMs represent a primary culture system; they do not reproduce *in vitro*. PAMs adhere readily to plastic culture surfaces and thus can be easily purified. In addition, a wide range of media have been employed successfully to culture PAMs. All of these culturing characteristics make an attractive model system for

studies of α-TH.

The purpose of this project is to determine appropriate PAM culture conditions for the study of α-TH turnover and to characterize the turnover of α-TH in culture. The primary objective for developing culturing conditions is to evaluate basal media types and serum concentrations for their effects on PAM viability and the status of α-TH. The characterization of α-TH disposition in culture requires the application of analytical techniques to this system and represents the second objective. The third objective of this research is to describe the contribution of physical exchange of unoxidized α-TH to α-TH turnover in culture. Finally, the effect of oxidative stress on PAM-associated α-TH will be studied in PAM activated with PMA.

CHAPTER 2

METHODS

MACROPHAGE ISOLATION

Alveolar macrophages were isolated from 300-400 g male Sprague-Dawley rats (Sasco, Omaha, NE). The animals were housed in hanging cages without bedding material for up to three weeks. Animals were anesthetized with 65 mg/kg pentobarbital. After reflexes were absent, the trachea was exposed and cannulated with .062" O.D. polyethylene tubing affixed to an 18-gauge needle. Lungs were inflated in situ with 5 ml of 37°C sterile lavage fluid (145mM NaCl, 5mM KCl, 1.9mM $NaH_2PO_4 \bullet H_2O$, 9.35mM Na_2HPO_4, 5mM glucose) then fluid was withdrawn as the thoracic cavity was gently massaged. Lavage was repeated 10 times for each animal and the lung fluid was collected in sterile 50 ml polyethylene conical tubes on ice. These tubes then were centrifuged at 3200 rpm for 12 minutes at room temperature and the cell pellets were washed twice with 30 ml of Ham's F12 medium (Gibco, Grand Island, NY) serum free with 1.176g/l $NaHCO_3$ and 1% Penicillin/streptomycin (Gibco). The washed cell suspensions were combined and then cell counts and viability were determined by eosin red exclusion with a hemocytometer. Equal volumes (200 μl) of cell suspension and eosin red were combined and allowed to incubate for 5 minutes. Both chambers of the hemocytometer were filled with this mixture and 10 quadrants were counted. The average number of macrophages per quadrant was multiplied by a dilution factor of 2 and

by 5 X 10^4 to evaluate the number of cells per milliliter of cell suspension. The cell suspension was then diluted with Ham's F12 to 4 X 10^6 cells per milliliter of suspension.

CELL CULTURE

All cell cultures were plated at a density of 4 million cells per 35 mm plastic tissue culture dish (Corning, Corning, NY) except in density experiments. One milliliter of the previously described cell suspension was placed in each dish and cells then were allowed to attach for 30 minutes in a humidified incubator at 37°C and 5% CO_2. Two basal media were used to culture macrophage, RPMI 1640 (Gibco) w/2.0 g $NaHCO_3$ and 1% penicillin/streptomycin; and Ham's F12 (Gibco). These media were supplemented with varying concentrations of fetal bovine serum (FBS) (Gemini, Calabasas, CA). After macrophages attached to the dish, cultures were purified by vigorously washing each dish 3 times with 2 ml volumes of 37°C plating medium which varied with experimental conditions. Each culture was plated in 1.5 ml of plating medium and incubated at 37°C and 5% CO_2 for up to 3 weeks. For experiments lasting more than 4 days, medium was changed every 3 days.

Culture Viability

Viability of cultured macrophages was determined by trypan blue exclusion of undisturbed cultures. The culture medium was removed carefully with a pasteur pipet and then 300 μl of 0.4% trypan blue in 0.85% saline (Gibco) was added. Macrophages

were incubated with trypan blue for 5 minutes at room temperature and then observed with an Olympus BH-2 microscope with a 40X objective. Cell counts were made from 5 random fields and the ratio of unstained cells to total cell number was calculated.

Culture Purity

The purity of alveolar macrophage in culture was determined by esterase staining. Diagnostic staining kits for naphthol AS-D chloroacetate esterase (Sigma, St Louis, MO) and α-naphthyl acetate esterase (Sigma) were employed as a double staining technique for monocytes. Cultures were fixed by immersion into a citrate-acetone-formaldehyde fixative for 30 seconds then rinsed and stained in α-naphthyl acetate solution at 37°C for 30 minutes. The stained cultures then were rinsed a second time and stained with naphthol AS-D chloroacetate solution at 37°C for 15 minutes. Double stained cultures were rinsed once again then counter stained in Hematoxylin for 2 minutes at room temperature. After a final rinse, cultures were allowed to air dry and were evaluated microscopically. Monocytes, including macrophages, appeared as black granulated cells whereas other cell types have red or no visible granulation.

Culture Protein Content

The protein content of macrophage cultures was determined by the Pierce BCA protein assay (Pierce, Rockford, IL). One day old cultures were taken from the incubator and the medium was removed from each. The macrophages were then scraped

up in 0.75 ml of phosphate buffered saline (PBS) and an additional 0.4 ml of PBS was used to rinse the plate. The scraped cell suspension and rinse were combined for a final volume of 1.15 ml. The working reagent was prepared by mixing 10 ml reagent A (BCA detection reagent in buffer) with 0.2 ml reagent B (4% copper sulfate). A 100 μl aliquot of cell suspension was added to a tube with 2 ml of working reagent and then incubated for 30 minutes at 37°C. After the incubation, the tubes were allowed to cool and the absorbance at 562 nm was measured with a Hewlett Packard 8452A diode array UV-vis spectrometer with quartz cuvettes. The protein concentration was calculated using bovine serum albumin (BSA) standards.

Macrophage Activation

Macrophage cultures were activated with the phorbol ester, phorbol-13-myristate-12-acetate (PMA) (Sigma). A 1 mg/ml solution of PMA was prepared in DMSO (Sigma) and 20 μl aliquots of this solution were stored at -20°C. At time of use, an aliquot of PMA was thawed and diluted with 180 μl of RPMI containing 5% FBS. Randomly selected cultures, had their medium removed gently with a Pasteur pipet and 30 μl of the PMA solution was directly applied over the cells. These cultures were allowed to incubate for 5 minutes and then 1.5 ml of RPMI containing 5% FBS were added gently to the culture dish.

Inhibition of Superoxide Production

Superoxide production by macrophages was inhibited by treatment with 10 mM iodoacetate. The iodoacetate solution was prepared by adding 55.8 mg of iodoacetic acid to 2 ml RPMI with 5% FBS and then adjusting the pH to 7.4 with 30 μl of 5N NaOH. Randomly chosen cultures were treated by adding 100 μl of this solution to the 1.5 ml of plating medium for a final concentration of 10 mM iodoacetate.

Inhibition of Nitric Oxide Production

Macrophages production of NO was inhibited by treatment with N^G-monomethyl-L-Arginine (NMA) (Calbiochem, San Diego, CA). NMA solution was prepared by dissolving 25 mg NMA in 2 ml RPMI with 5% FBS. Selected cultures were treated with 100 μl of NMA solution to achieve a final NMA concentration of 3.36 mM.

Vitamin E Assay

Culture medium was removed with a silanized Pasteur pipet and transferred to a silanized culture tube. The macrophages then were scraped up in 0.75ml of PBS and an additional 0.4 ml PBS was used to rinse the culture plate. Both the scraped cells and the rinse were combined in a silanized culture tube. To each sample of medium or cells, 100 μl of 1 μM δ-tocopherol (δ-TH; Sigma) was added as an internal standard along with 25 μl 1N BHT (Sigma), 1 ml 10 mM lauryl sulfate (SDS)(Sigma) and 2 ml ethanol. This mixture was vortexed for 10 seconds and then sonicated for an additional 10 seconds with

a Heat Systems Ultrasonics, W-380 ultrasonicator equipped with micro tip. Samples were then extracted twice with 2 ml hexane and the extracts were combined and evaporated to dryness under nitrogen.

The dry residue was dissolved in 0.5 ml of methanol and samples then were transferred to 1 ml autosampler vials for HPLC analysis. Injections of 0.1 ml were made with a Spectra Physics SP8775 Autosampler. Tocopherols were resolved on a 4.6x 150 mm spherisorb ODS-2 column (Alltech, Deerfield, IL) eluted with methanol/1M sodium acetate (98:2,v/v). The mobile phase was delivered at a flow rate of 1.5 ml min^{-1} with a Spectra Physics model SP8810 isocratic pump. Tocopherols were detected with a model 5100A electrochemical detector (ESA, Bedford, MA) equipped with a model 5010 analytical cell. Tocopherols were detected by oxidation at an electrode potential of +35 volts. Chromatograms were recorded with a Spectra Physics SP4600 integrator interfaced with a Heath-Zenith 286 computer equipped with WINner chromatography software (Spectra Physics, San Jose, Ca). The concentrations of α-TH were calculated by the internal standard method from the ratio of α-TH peak area to δ-TH peak area.

Superoxide Production

Superoxide production by cultured macrophages was measured as the superoxide dismutase-inhibitable reduction of cytochrome c. Medium was removed from cultures and 1 ml of a 1 mg/ml cytochrome c type IV (Sigma) in phosphate-free Krebs-Hepes buffer (25 mM hepes, 6.87 g/l NaCl, 0.4 g/l KCl, 0.14 g/l $MgSO_4 \cdot H_2O$, 2.1 g/l

$NaHCO_3$ and 2.0 g/l glucose) was then added. Control cultures were pretreated with 1 ml of a 0.4 mg/ml superoxide dismutase (SOD)(Sigma) solution in Krebs-Hepes buffer. After a 3 minute incubation at room temperature, 1 ml of 2 mg/ml cytochrome c was added to the cultures containing SOD and then all cultures were incubated in the dark for 10 minutes at 37°C with gentle agitation. Supernatant from each culture then was placed in a plastic microcuvette and the absorbance at 550 nm was measured with a Hewlett Packard 8452A spectrophotometer. Superoxide concentration was calculated from the difference in absorbance between experimental cultures and SOD treated cultures. Calculations were based upon a 1:1 molar relationship between cytochrome c and O_2^-. The concentration was determined with an extinction of 21.1 mM^{-1} cm^{-1}.

Nitric Oxide Production

NO production was estimated by the formation of nitrite, a NO oxidation product, in the culture medium. Nitrite was measured colorometrically using the Greiss reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride and 5% H_3PO_4) which forms a chromophore that absorbs at 543 nm. A 200 μl sample of culture medium was combined with 600 μl of the Greiss reagent and then the absorbance was measured at 550 nm with a Hewlett Packard 8452A diode array spectrophotometer.

Statistical Analysis

Statistical significance was determined by two-tailed student-t test. Differences

were considered significant for p values < 0.05. Data are presented as the mean value $\pm$ standard deviation.

CHAPTER 3

RESULTS

Evaluation of Cell Culture Conditions

The initial goal of this project was to determine optimal culture conditions for PAM for studies of α-TH turnover. To accomplish this goal, two basal media were selected to screen culture conditions. The first was RPMI 1640, a complete medium used extensively for culturing PAM. In addition to its rich complement of amino acids and vitamins, RPMI 1640 is supplemented with 1 mg/l GSH. The second medium used was Ham's F12 nutrient mixture. This medium has a complete complement of amino acids, just as RPMI. However, concentrations of most are 50-75% of those in the RPMI. The F12 medium is not supplemented with GSH; instead it has supplements of hypoxanthine, linoleic acid, lipoic acid, putrescine, sodium pyruvate and thymidine. This medium is also enriched with 13.96 mg/l choline chloride and 1.36 mg/l vitamin B_{12}; RPMI has 3 mg/l and .005 mg/l, respectively.

Cultures were originally plated at a density of 3 million cells per 35 mm culture dish. The cells remained in culture for up to 3 weeks with medium changes every 3 days. Cultures were assayed for α-TH and assessed for morphologic appearance. Both media were used with supplements of either 0, 1 or 5% FBS. Cultures grown in serum free HAMs F12 medium had the best long term cell attachment and healthy appearance. Serum free RPMI 1640 consistently caused poor cell attachment to culture dishes and the

appearance of those cells that remained attached was rough and granulated without prominent membranes. Incorporation of serum into both media types led to high plating efficiencies and smooth and phase-bright appearances with well spread and defined membranes. Cultures grown in RPMI 1640 with serum often became contaminated by proliferation of other cell types after 6-8 days in culture. When this occurred, contaminating cells usually overgrew plates within 2-3 days. Cultures plated in Hams F12 did not become contaminated with other proliferating cells.

One 35 mm petri dish of cells was used for each α-TH analysis. At 3 million PAM per plate, α-TH was easily detected in freshly isolated cells, but α-TH became difficult to measure in the remaining adherent cells. Therefore, the plating density was increased to 4 million PAM per plate. At this density, cells appeared almost confluent in this size dish. All subsequent experiments were performed at a density of 4 million PAM per plate. All serum free cultures were maintained in Hams F12, whereas the serum-supplemented medium was RPMI 1640.

α-TH DEPLETION IN PAM CULTURE

The dominant feature of α-TH status in PAM cell culture was a rapid depletion of cellular α-TH levels in the first 24 hours (Figure 7A). This depletion was accompanied by the appearance of significant amounts of α-TH in the medium (Figure 7B). The zero time point was taken 30 minutes after first PAM were seeded. Then plates were washed and analyzed for α-TH. This procedure allowed the removal of

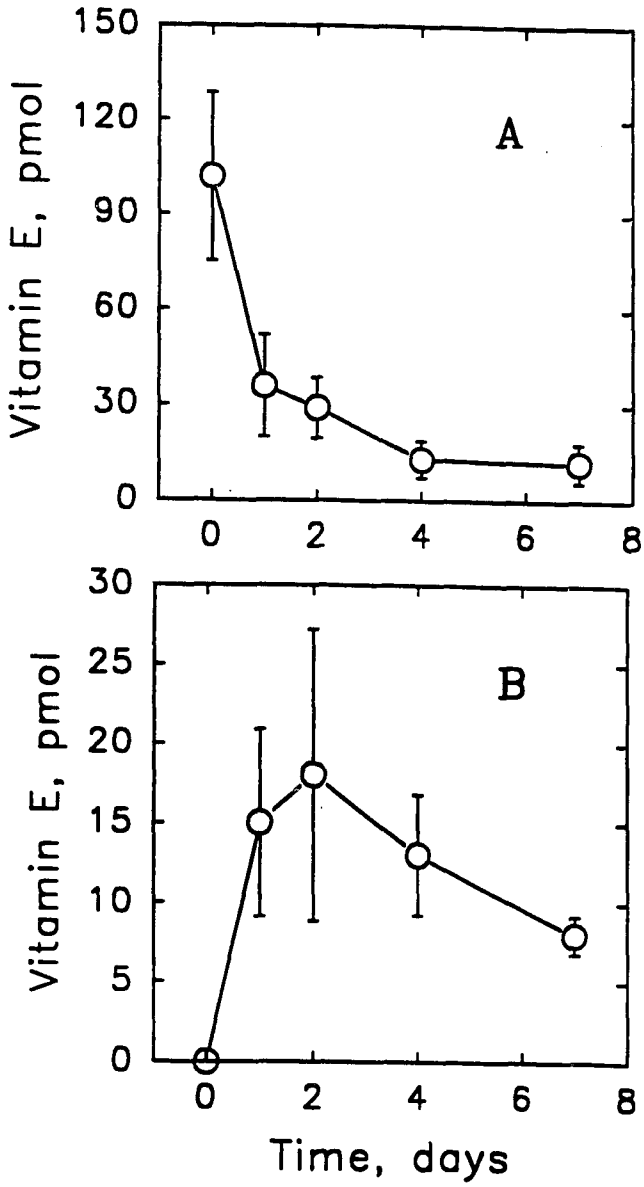


Figure 7. (A) Cellular concentrations of α-TH in 4 x 10^6 PAM versus time. (B) medium (RPMI w/ 5% FBS) concentrations of α-TH from the same cultures. All data combined from 5 experiments over 7 months. All values expressed as means $\pm$ SD.

contaminating cell types and unattached macrophages prior to α-TH analysis. Significant numbers of unattached PAM always were washed away, so cultures always were inspected visually for consistent appearance with respect to plating density. PAM remained in the same medium for the remainder of the experiments. At the initial time point, the average α-TH level was 102 ± 27 picomoles per 4 million PAM. The data in Figure 7 were obtained from 5 experiments done over a 7 month period in which triplicate analyses were done at each time point. The standard deviations reflect the variability between platings. Initial levels of α-TH ranged from 40 pmoles to 240 pmoles per sample. The deviation within platings was much less and averaged about 20 pmoles. Regardless of the initial level of α-TH, the drastic decrease of cellular α-TH levels was consistent.

The data in Figure 7 was obtained from cultures grown in RPMI 1640 with 5% FBS. In all cultures grown in the presence of serum, the decrease of cellular α-TH was always accompanied by the appearance of unoxidized α-TH in the medium. Once again, there was a high degree of variability between experiments that paralleled the cellular levels of α-TH. On the average, 16 ± 6 pmoles of unoxidized α-TH appeared in medium in the first 24 hours. This accounts for 25% of the α-TH lost by the PAM. These experiments were carried out in two media types, RPMI with 5% FBS and Hams F12 without serum supplement. A similar loss of cellular α-TH over the first 24 hours occurred in cultures grown in the serum free F12 medium, however no α-TH was detected in the medium (data not shown). These experiments demonstrate that α-TH is

rapidly lost from cellular stores and that a portion is released to the culture medium as unoxidized α-TH.

Additional experiments were needed to determine if viability of cells, purity of cultures, and consistency of sample preparation had any relationship to the behavior of α-TH in culture. Three experiments were performed to examine the viability of PAM as a function of time without medium changes. Quantification of the appearance of α-TH in the medium also required an evaluation of viability of cells in the same medium. Figure 8 demonstrates that PAMs cultured in RPMI with 5% FBS had an initial decrease in viability of 12-15% in the first 24 hours. Viability in these experiments was determined by trypan blue exclusion. The 65% drop in cellular α-TH was accompanied by 15% decrease in viability. After this decrease, cell viability remained constant for the first week. Between 7-9 days there began a rapid decrease in viability that continued for the duration of the experiments. Similar results were seen in cultures grown in Hams F12 without FBS, viabilities were 85-88% for the first week and then dropped.

The purity of PAM in cell culture was determined by double staining of 2 esterase enzymes specific for monocytes. These stains were performed periodically on 1 day old cultures over a 6 month period. All of the cultures assayed contained >95% positively stained cells which indicated high purity of monocytes. This assay, however, is not effective in differentiating between tissue macrophages and other monocytes or lymphocytes.

To extract vitamin E from PAMs, cells must be physically scraped from the

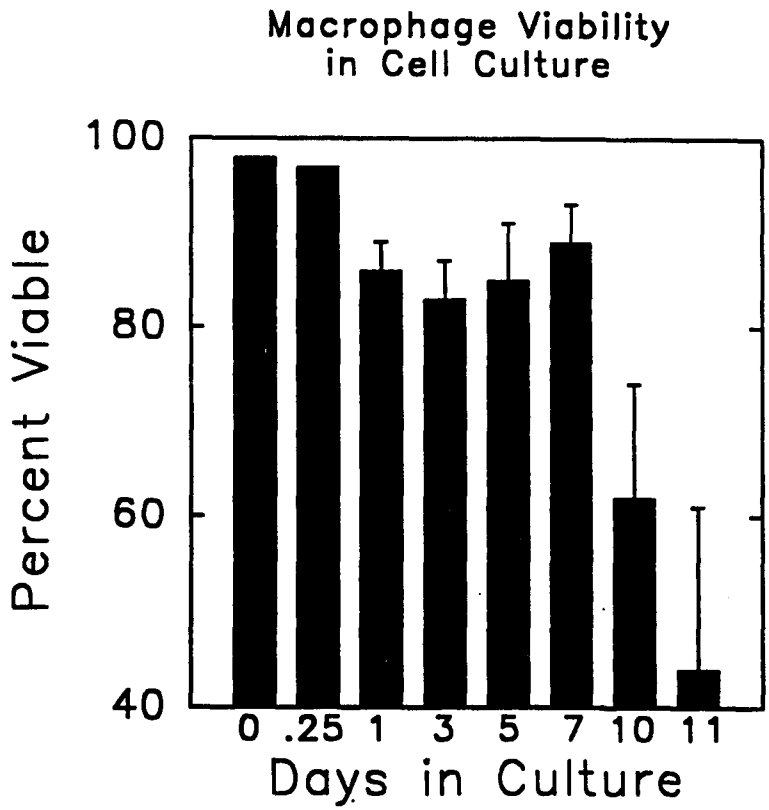


Figure 8. Viability of cultured PAM versus time in RPMI w/ 5% FBS. Viability was determined by trypan blue exclusion. Data combined from 3 experiments where 1 culture from each time point had 250 - 300 cells counted from 5 fields of view.

surface of the culture plastic. The consistency with which cultured PAM were resuspended was determined by protein content. Protein assay was performed in triplicate on 2 sets of cultures that were plated 6 months apart. The average protein content was 0.496 ± .025 mg per 4 million cells. These results suggest good reproduciblity in the harvest of cell samples within and between platings.

Once viability, purity, and consistency of cultures had been established, other aspects of the culture system were examined for their effect on α-TH disposition. Plating density presented a concern because of the relatively crowded conditions within the culture dish. To address this concern, an experiment was performed to study the kinetics of α-TH loss of in cultures of 1 million, 2 million and 4 million macrophages. This experiment indicated that all cultures decreased to the same level in 2 days (data not shown). Although the high density cultures lost α-TH at a greater rate, the fractional loss of cellular α-TH was similar at all plating densities.

DISPOSITION OF RELEASED α-TH

The observation that α-TH did not appear in serum free medium focused the interest in media composition. A general hypothesis is that some component of the serum is acting as a carrier for the highly lipophilic α-TH. Possible carriers in serum include lipoproteins which carry α-TH in the plasma *in vivo*. This α-TH-serum complex may solubilize α-TH in the aqueous phase, which allows α-TH to be detected in the medium. Therefore, the amount of unoxidized α-TH detected in the medium may vary

directly with serum content. Figure 9 shows data from two experiments comparing serum content and α-TH recovery in the medium. These experiments compared levels of α-TH found in the medium from cultures maintained at two different serum concentrations. Cells were cultured in RPMI with either 5% or 10% FBS. Serum free cultures were performed in Ham F12 medium because of poor plating efficiencies in serum free RPMI medium. To ensure that differences in the basal media types could not account for the appearance of α-TH in the medium, cultures were plated in RPMI and Ham F12 both with 5% FBS. In all the media types, a similar rate of α-TH depletion was observed (Figure 9A). RPMI with 5% FBS appeared to maintain the highest cellular α-TH concentrations at 1-4 days.

Figure 9B illustrates the appearance of α-TH in the different media. By the second day in culture, the medium with 10% FBS supplement had shown an increase in α-TH greater than the other media types. After 4 days, this increase was larger, however it occurred long after the cellular depletion had occurred. Similar levels of α-TH were recovered in both RPMI and Ham F12 when supplemented with 5% FBS. All subsequent culturing was done in RPMI with 5% FBS, unless serum-free conditions are stipulated.

These results appear to demonstrate that increases in serum content can increase the recovery of α-TH from the medium, possibly by increasing the amount of carrier for α-TH association. The sustained increase in medium α-TH levels after cellular α-TH depletion suggests that there may be substrate(s) other than serum components for α-TH

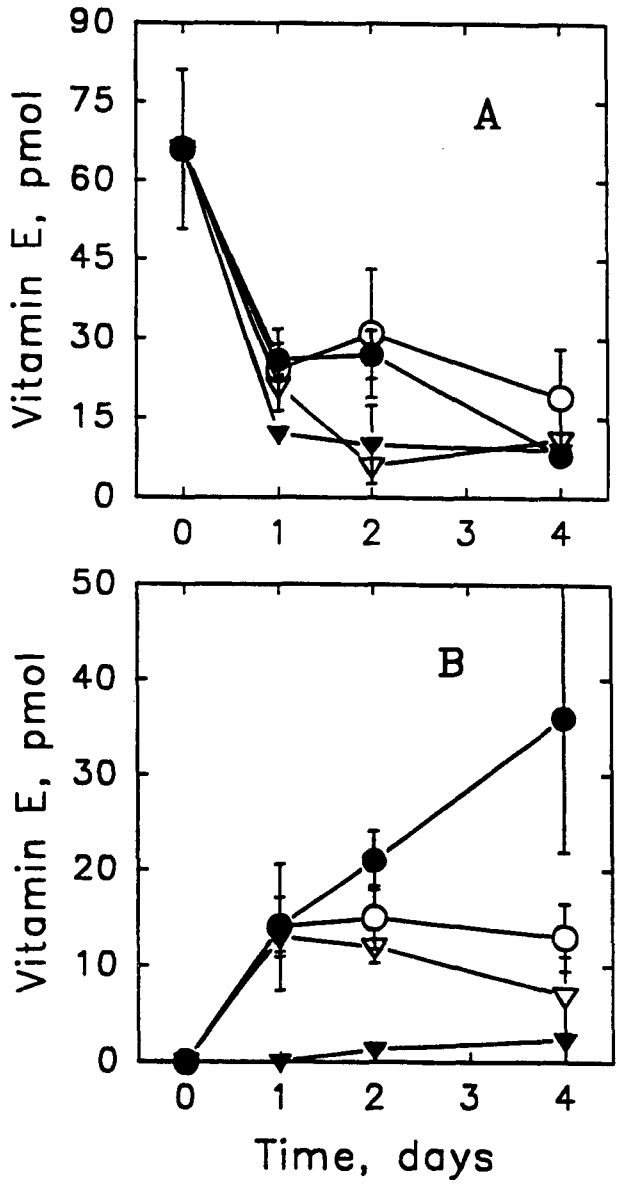


Figure 9. Effect of medium composition on α-TH loss from PAM and appearance in medium. Four medium types were examined. RPMI w/ 10% FBS (●), RPMI w/ 5% FBS (○), Ham F12 w/ 5% FBS (▽) and Ham F12 w/o FBS (▼). (A) Cellular levels versus time and (B) medium levels versus time. Data from 2 experiments in triplicate, n=6. All values are expressed as mean ± SD.

association. We examined the hypothesis that α-TH could also associate with the surface of the culture dish. To test this hypothesis, several experiments were performed. First, direct extraction of α-TH from the culture surface was attempted after the removal of cells. A variety of organic solvents were tried without success. More polar solvents, such as methanol and ethanol did not yield any α-TH after extensive washes. Less polar solvents, such as ethyl acetate dissolved the culture surface. Consequently, indirect methods for determining α-TH-culture surface interaction were explored.

The first goal was to determine if the culture surface could bind α-TH. A 100 pmol sample of α-TH in 25 μl of methanol was applied to a series of culture dishes. After evaporation of the solvent, 1.5 ml of RPMI serum containing medium or Ham F12 medium was placed in each dish. The dishes were incubated for a period of 4 days with daily medium changes. This medium was analyzed daily for the appearance of α-TH. No α-TH was detected in any of these medium samples. These experiments suggest that the surface of the culture dish can bind α-TH.

Next we studied the depletion of α-TH from samples of RPMI medium containing 5% FBS and spiked with α-TH. To culture dishes containing 1.5 ml of medium was added 50 pmol of α-TH in 25 μl of methanol. Figure 10 illustrates the persistence of α-TH in medium with time. The initial α-TH value (50 pmol) was the amount of α-TH used to spike the medium. The time zero level was consistently around 30 pmol which indicates that not all the α-TH added to the medium can be recovered. The increase at the 1 hour time point was also consistent. This may indicate an initial association with

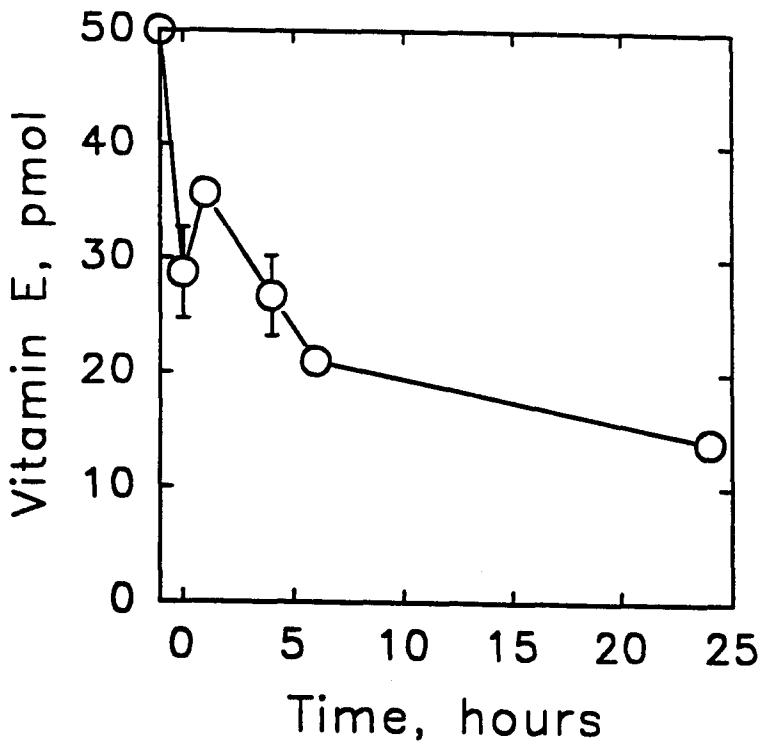


Figure 10. Disappearance of α-TH from RPMI w/ 5% FBS. The first point represents the 50 pmol of α-TH added to each culture dish with 1.5 ml medium. The second point represents the concentration measured immediately after the addition of α-TH to the medium. The data were combined from 2 experiments, n=6. All values are expressed as mean ± SD.

the plate that does not completely inhibit α-TH from moving to the medium. From 1-6 hours there is a 42% decrease of α-TH recovered from the medium. After 24 hours, the α-TH concentration appeared to have stabilized at about 14 pmol. These experiments imply that there may be some competition for α-TH between the culture surface and the medium.

Since the plate and the medium apparently, can compete for α-TH, more α-TH may be released than can be accounted for in the medium at any one time. An experiment was designed to remove α-TH from the culture system as it was released by the PAM. Cultured PAM were divided into 2 sets. The first set was plated in 1.5 ml of RPMI with 5% FBS and remained in the same medium for the duration of the experiment. These cultures were termed control cultures. The second set was plated in the same medium; however this medium was changed at various intervals. These medium changes are termed "washes" and this set of cultures was referred to as "washed cultures". Each of these washes was analyzed for α-TH at every interval. Control and washed PAMs were analyzed for α-TH at 12, 24, 42 and 96 hours. Levels of α-TH in control medium were also taken at these times. Figure 11A illustrates the depletion of cellular α-TH from both control and washed PAMs. The time course of α-TH loss was virtually identical for both culture conditions. In Figure 11B, the control values represent the level of α-TH in the medium at each time point. The values for medium washes, however, represent the cumulative amount of α-TH removed from the culture system. After 4 days, 75 $\pm$ 11 pmol of α-TH were recovered in the medium of the

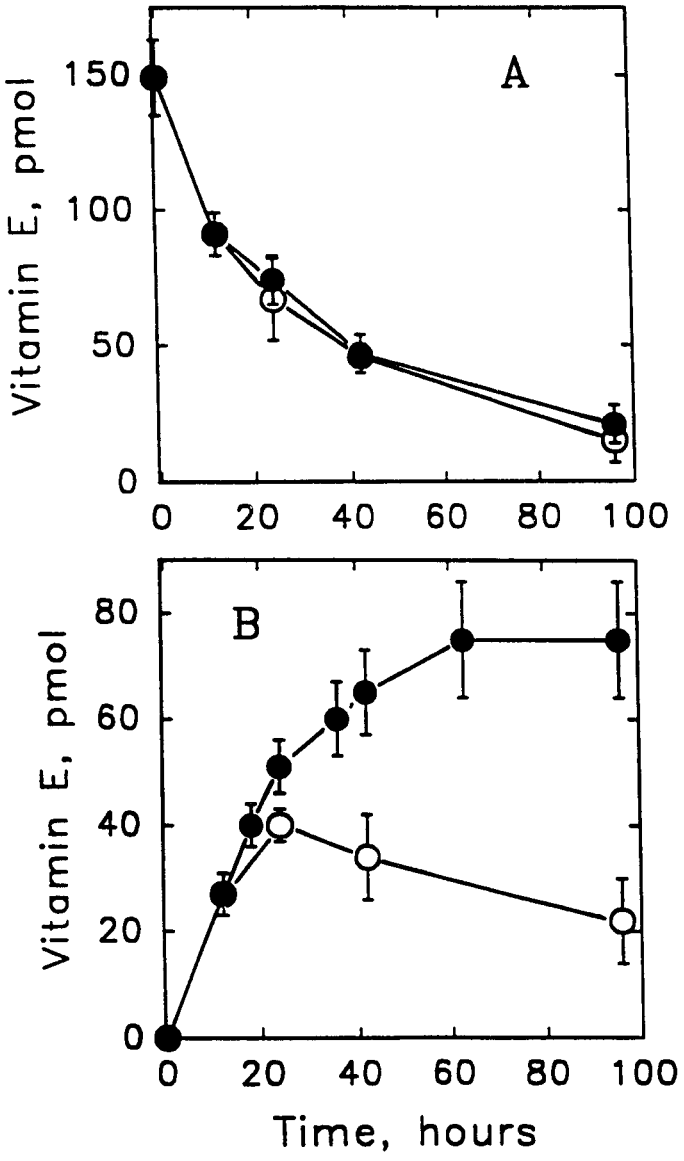


Figure 11. Determination of the amount of α-TH released by PAM was achieved by repeated removal of medium. PAM cultured in RPMI w/ 5% FBS had either repeated medium changes (●) or remained in the same medium for the duration of the experiment (○). (A) Cellular levels show no differences between treatment groups. (B) Medium levels show that by adding together α-TH levels from each change, more α-TH could be accounted for in the medium. Data was from 1 experiment done in triplicate, all values expressed as mean ± SD.

washed cultures. This accounts for 59% of the α-TH depleted from the PAMs over this period of time. The 22 ± 8 pmoles of α-TH detected in the control culture on the fourth day only accounts for 16% of the depleted α-TH. The medium change had no effect on the kinetics of α-TH depletion, since the time course of cellular α-TH content was identical in control and washed cultures. This experiment confirms that more α-TH is released than can be measured at any one time point.

In the previous experiment, the greatest loss of α-TH occurred in the first 12 hours. Of the 58 pmol lost from the PAMs, only 47% of α-TH appeared in the medium at this time. Additional experiments were performed to determine how much of this initial decrease was due to release of α-TH. Using the same protocol as previously described, washes and culture samples were taken at 1, 3, 6, 9, and 12 hours. On the average, PAMs lost 35 pmol of α-TH over the first 12 hours. 63% of this loss could be accounted for in the medium washes. In conclusion, these experiments demonstrate that release of unoxidized α-TH accounts for about 60% of the α-TH turnover in cultured macrophages.

VITAMIN E UPTAKE EXPERIMENTS

In early experiments, cultures were sampled daily for α-TH and the cellular level of α-TH appeared to be rapidly and steadily declining. As the dynamics of α-TH release became apparent, sampling intervals were decreased to every 6 or 12 hours. These shorter intervals between samples lead to an interesting finding. In about half of the

experiments, after the initial rapid release of α-TH had slowed (i.e. at about 12 hours), cellular levels of α-TH appeared to increase. This increase was usually seen between 18 and 24 hours after plating. It was hypothesized that PAM may reabsorb α-TH from the medium. To study this phenomenon, an experiment was initiated with two sets of PAM cultured for 48 hours with α-TH samples taken every 6 hours. The first set was plated in RPMI with 5% FBS medium, whereas the second was plated in Ham F12 serum free medium. Figure 12A shows cellular α-TH levels for the RPMI and Ham F12 cultures. Cellular α-TH steadily decreased for 18 hours and levels fell to 21 ± 5 pmol. However, α-TH levels in PAM cultured in the presence of serum then increased at 24, 30, and 36 hours. The increases were all between 3 and 6 pmoles, none of which were statistically significant over the value of the 18 hour time point. In PAM cultured without serum, α-TH steadily decreased until α-TH was virtually depleted at 30 hours. The levels of α-TH in medium samples for both culture conditions is illustrated in Figure 12B. As observed previously (see above), α-TH lost from PAM did not appear in the serum free medium. Because of the low initial concentration of α-TH, this experiment was repeated. A similar pattern of α-TH depletion and apparent reuptake was again observed but once again the magnitude of the reuptake was not statistically significant (data not shown). These data illustrate that in the presence of serum cellular levels of α-TH are better maintained.

The above observations suggest that PAM could be "loaded" with α-TH. Previous results (see above), suggest that α-TH associates with components of serum in

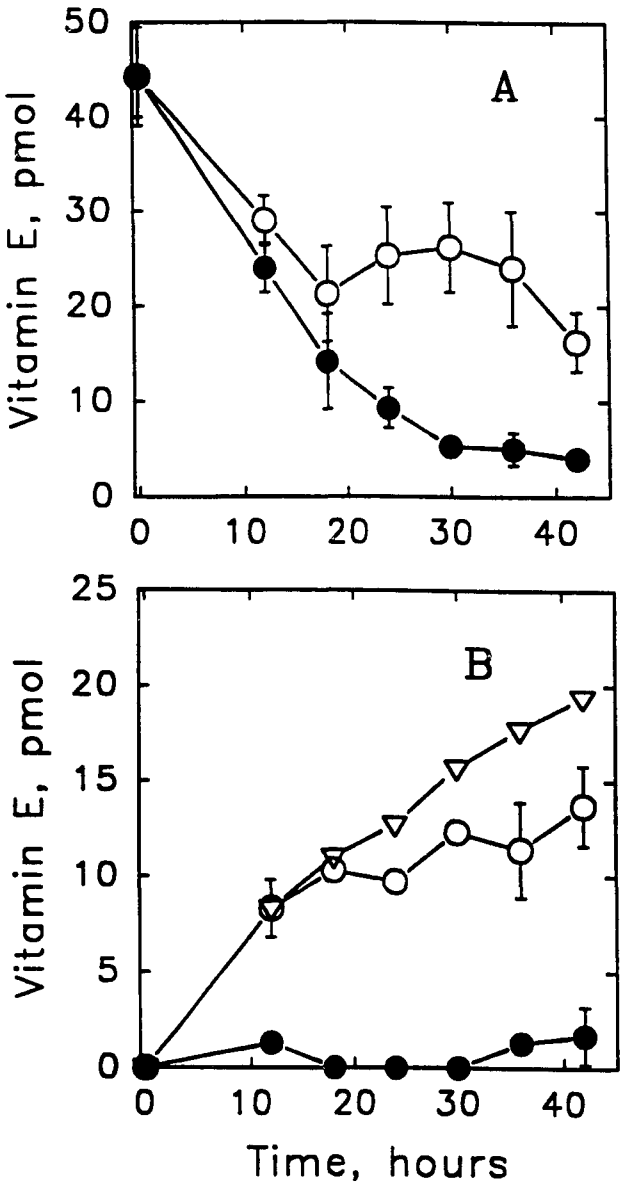


Figure 12. Resorption of α-TH from the medium was determined by short sample intervals from PAM cultures in either RPMI w/ 5% FBS (○) or in serum-free Ham F12 (●). (A) Cellular α-TH levels shows an increase at 24 hours in RPMI w/ 5% FBS, while Ham F12 shows continued depletion. (B) Medium levels show serum must be present for uptake to occur and medium changes account for an additional 6 pmoles of α-TH in the medium (▽). Data represents 1 experiment done in triplicate. All values are expressed as mean ± sd.

the culture medium. Without the presence of serum, α-TH does not appear in the culture medium and PAM uptake of α-TH does not occur. If the amount of α-TH in serum could be greatly enhanced, then the amount available for PAM uptake from the medium likewise could be increased. A protocol developed by Esterbauer *et al.* (1991), which described the loading of LDL with α-TH, was used to enrich FBS with α-TH at 100 times the naturally occurring level. This α-TH enriched serum was used to supplement RPMI at the usual 5% total serum content. PAM were cultured in Ham F12 serum free medium for 6 days. At the end of 6 days, cellular α-TH levels had been depleted to 9 pmol. The culture medium was replaced with RPMI containing 5% α-TH enriched FBS and cellular levels of α-TH then were measured at 6 and 12 hours. The culture medium contained approximately 400 pmol of α-TH in each culture dish. Figure 13 shows that PAM α-TH levels increased to 54 ± 7 pmol after 6 hours and then fell to 36 ± 9 pmol at 12 hours. These experiments support the hypothesis that PAM are able to receive α-TH from components of serum in the medium.

EFFECTS OF PAM ACTIVATION AND OXIDANTS ON α-TH STATUS

The above experiments have described the status of α-TH in resting cultured PAM. Under resting conditions, the most prominent component of α-TH turnover appears to be the release of unoxidized α-TH to the medium. Because the activation of PAM involves the production of free radicals and other oxidants, we addressed the question of how the PAM α-TH status would respond to oxidative stress.

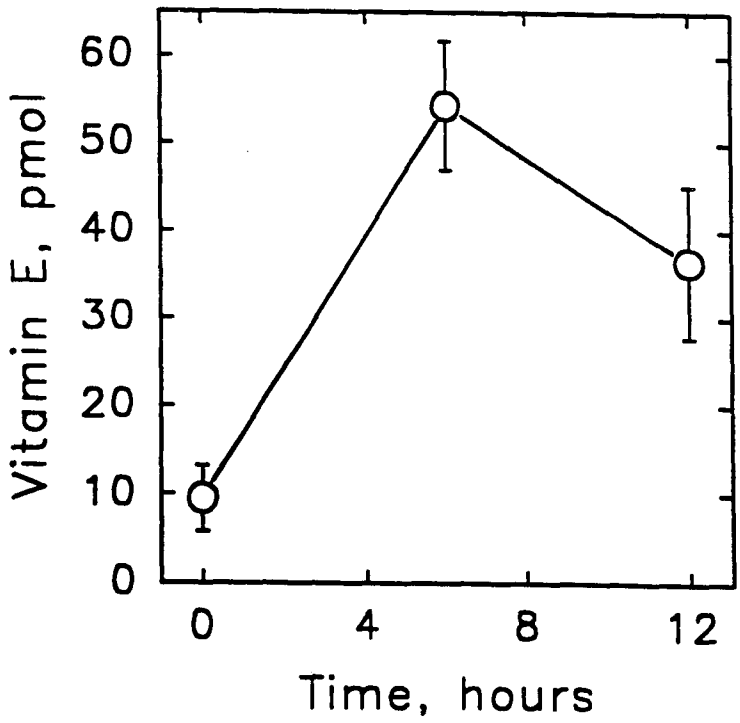


Figure 13. α-TH uptake was determined by culturing PAM in RPMI w/5% FBS enriched with 270 pmol/ml α-TH. Cellular levels increased dramatically over 6 hours. Data are from 1 experiment in triplicate. All values expressed as mean $\pm$ sd.

The first experiment involved the introduction of a O_2^- generating system to cultured PAMs. In this protocol, an exogenous oxidative stress was used to affect PAM, whereas in subsequent experiments, oxidative stress was induced by stimulation of PAM oxidant production (see below). O_2^- was generated by the addition of xanthine/xanthine oxidase, along with $FeCl_2$ as a Fenton catalyst, to cultured PAMs. Four sets of PAM were cultured in RPMI medium containing 5% FBS. The first set served as the untreated control group, whereas the other three were treated with either xanthine/xanthine oxidase, SOD or both. Cells were exposed to treatments immediately following plating/washing and allowed to incubate for 12 hours. Cellular and medium α-TH levels were assayed at 12, 18 and 24 hours. Figure 14A represents cellular α-TH and exhibits the typical decline in α-TH over the first 12 hours regardless of treatment. At 18 hours, an increase of cellular α-TH was seen in all the treatment groups except for the xanthine/xanthine oxidase-SOD group. This difference may be associated with an interaction between SOD and the radical generating system. The similar behavior between the other three treatment groups indicates that the treatment used did not effect cellular α-TH. The medium α-TH levels remained similar for all treatment groups throughout the experiment (Figure 14B). Although α-TH status did not change, there were significant differences in the appearance of these cultures. Cultures treated with xanthine/xanthine oxidase had cells with membrane blebbing and extremely rough surfaces. There were large numbers of vacuoles in these PAM and their cytoplasm appeared extremely grainy. Any cultures treated with SOD had a smoother, more

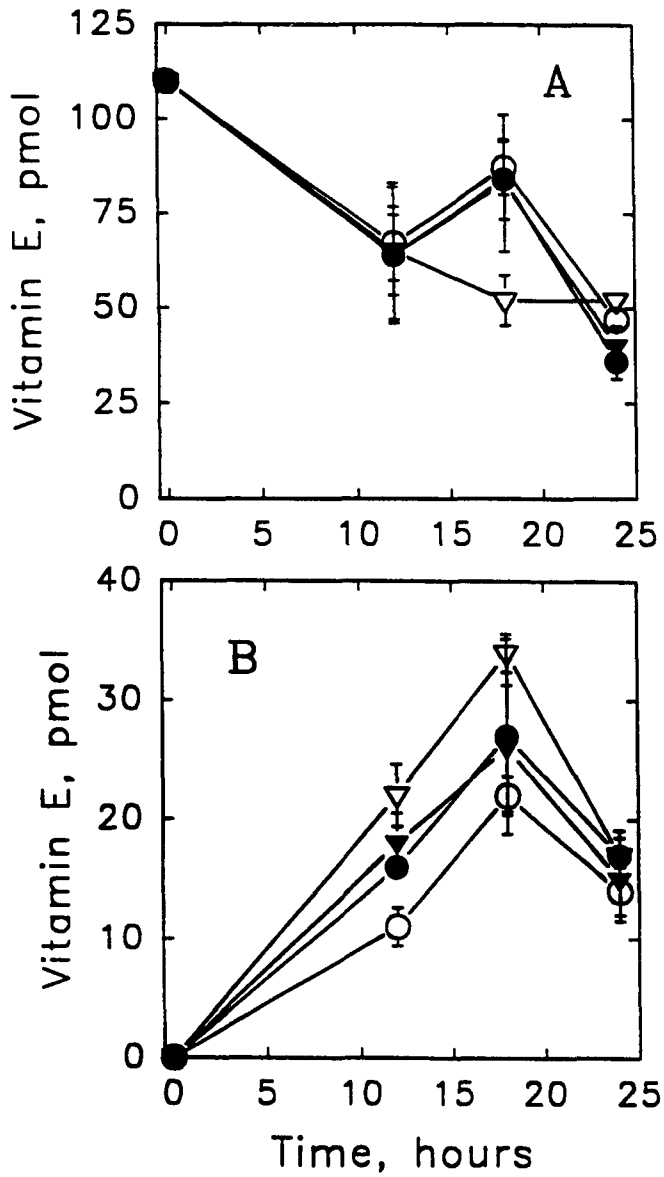


Figure 14. Effects of an exogenous radical generating system were examined in four treatment groups of PAM in RPMI w/5% FBS; untreated control (○); SOD treated (●); xanthine/xanthine oxidase treated (▼); and treated with both (▽). (A) Cellular levels appeared unaffected by treatment and (B) medium levels also were unaffected. Data represents 1 experiment done in triplicate. All values are expressed as mean ± SD.

regular appearance and well defined membranes. The differences in appearance between treatment groups indicates that the xanthine/xanthine oxidase had an effect upon the PAM.

The second group of experiments were done to investigate the effect of PAM activation upon the behavior of α-TH in culture. PAMs generate O_2^- in response to a variety of stimuli. PMA is a common phorbol ester used to stimulate O_2^- production in cultured PAMs. This stimulus initiates a cascade response that results in the activation of NADPH oxidase. There are no direct inhibitors of this oxidase enzyme, however depletion of its substrates may decrease its activity. Iodoacetate can be used to deplete NADPH in cultured cells, although its alkylating potential can produce other effects on PAM.

In these experiments, PAMs were divided into four treatment groups. A control group received no treatment; a second received PMA only; a third received PMA and iodoacetate; the fourth received iodoacetate only. The four groups were treated immediately following the plating and washing of cultures. Cellular and medium levels of α-TH were taken at 12, 18, and 24 hours after treatment. Figure 15A illustrates the behavior of cellular α-TH. All the treatment conditions demonstrated a similar decline of cellular α-TH up to 12 hours. The only treatment group to show a subsequent increase in α-TH at 18 hours was the group activated with PMA. Similar to previous experiments, this increase was not statistically significant from the previous time point. The increase also was not significant over the other treatment groups. These results

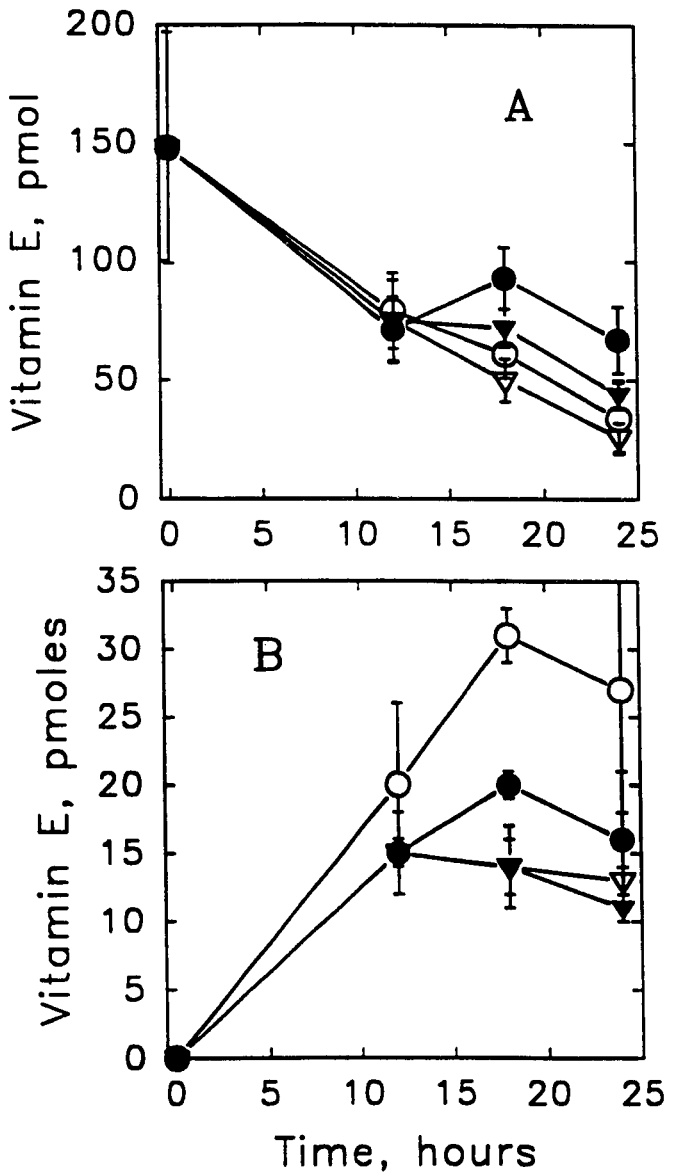


Figure 15. Effects of PMA activation on α-TH status was studied with four treatment groups of PAM cultured in RPMI w/ 5% FBS; untreated controls (○); treated with PMA (●); treated with iodoacetate (▼); and treated with both (▽). (A) Cellular α-TH levels and (B) medium α-TH levels versus time. Data are from 1 experiment done in triplicate, however results were duplicated 2 times. Because of differences in initial levels, data were not combined. The decrease in medium levels of PMA and iodoacetate treatment groups were reproducible. All values are means ± SD.

indicate that cellular depletion of α-TH was not affected by PAM activation.

Although cellular levels of α-TH did not appear to be affected by PMA stimulation, medium levels were noticeably affected (Figure 15B). Up to 31 ± 2 pmol of α-TH appear in the medium from untreated control cultures at 18 hour, which is well within the range observed previously (see above). In cultures treated with PMA, the medium α-TH level reached only 20 ± 1 pmol. This lower concentration was also seen in all cultures treated with iodoacetate, where the medium α-TH level at 18 hours was 15 ± 3 pmol.

The lower levels of α-TH seen in the medium from PMA treated cultures may have been caused by increased oxidation of α-TH after its release to the medium. This increase may be the result of increased radical production by the PAM. To test this hypothesis, superoxide production was measured in control, PMA and iodoacetate-treated PAM. Cultures were prepared in the same conditions as described in the previous experiment and individual culture dishes were assayed for superoxide at 0, 1, 3, and 6 hours. Samples were exposed to a cytochrome c solution for ten minutes and then cytochrome reduction was determined spectrophotometrically. Figure 16 shows that immediately after plating, all treatment groups were producing about 7 nmol of O_2^- per culture dish per 10 min. By the first hour in culture, untreated control cultures and iodoacetate treated cultures produced O_2^- much more slowly than PMA stimulated ones. At 3 hours, PMA stimulated cultures are still producing about 4.5 nmoles of O_2^- per 10 minutes, while iodoacetate treated and control cultures are producing no detectable O_2^-.

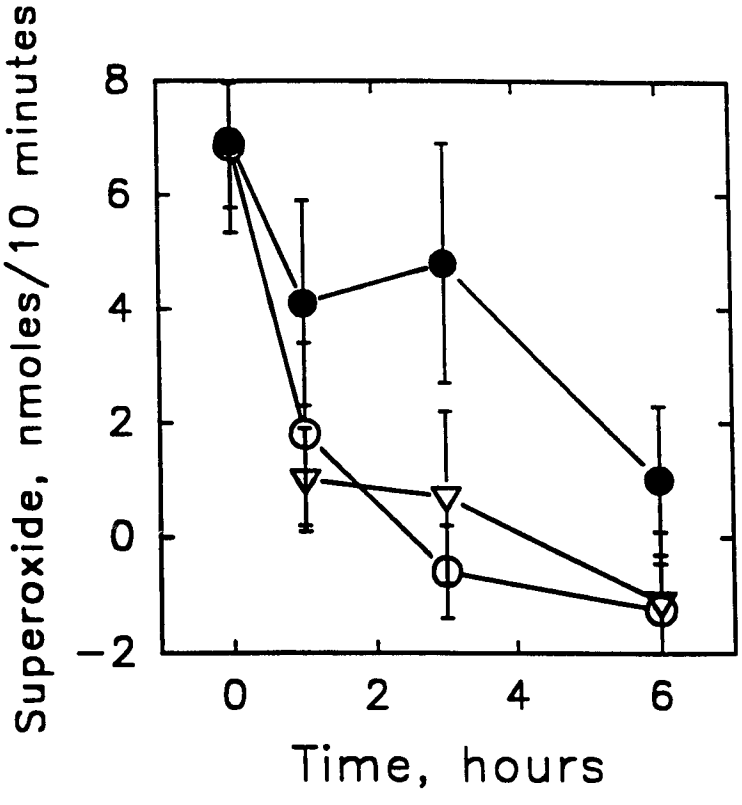


Figure 16. Determination of O_2^- production was performed using cytochrome-c. PAM in three treatment groups were assayed; untreated control (○); treated with PMA (●); and treated with iodoacetate (▽). Data is from 2 experiments performed in duplicate. Concentration of O_2^- was calculated from reduced cytochrome-c and its extinction coefficient (21.1 $mM^{-1}cm^{-1}$). All values are means ± SD.

Six hours after treatment, none of the treatment groups appeared to generate $O_2^{\cdot}$. These experiments confirm that iodoacetate can inhibit $O_2^{\cdot}$ production. Therefore, increased oxidation of α-TH by oxygen radicals is not a viable explanation for decreased levels in the medium of iodoacetate-treated cultures.

Another oxidant that may consume α-TH is NO, which is produced by cultured PAM following pretreatment with LPS or IFN-gamma. In our system, PAM were stimulated with PMA only. This treatment should not stimulate NO production, therefore NO should have no effect on the status of α-TH in culture. To address this question experimentally, we studied the effect of N^G-methyl-L-arginine (NMA) an inhibitor of PAM NO synthase. Four groups of PAM cultured in RPMI 1640 with 5% FBS were treated as follows. The first group (control) received no treatment; the second received PMA; the third received NMA; the fourth received both PMA and NMA. Figure 17 shows the PAM and medium levels of α-TH associated with each of the treatment groups. As observed previously, PMA had no effect on cellular α-TH status. NMA either alone or in combination with PMA did not affect the cellular α-TH depletion. Previously, cultures exposed to PMA had approximately half the amount of unoxidized α-TH in the medium as controls, 12 ± 3 pmoles vs 28 ± 4 pmoles. NMA treatment caused no changes in medium α-TH when compared with controls. Medium nitrite was measured to determine if PAMs were producing NO under these conditions. None of the treatment groups demonstrated any significant increase of nitrite over background levels. These results suggest that NO is not involved in the mechanism of

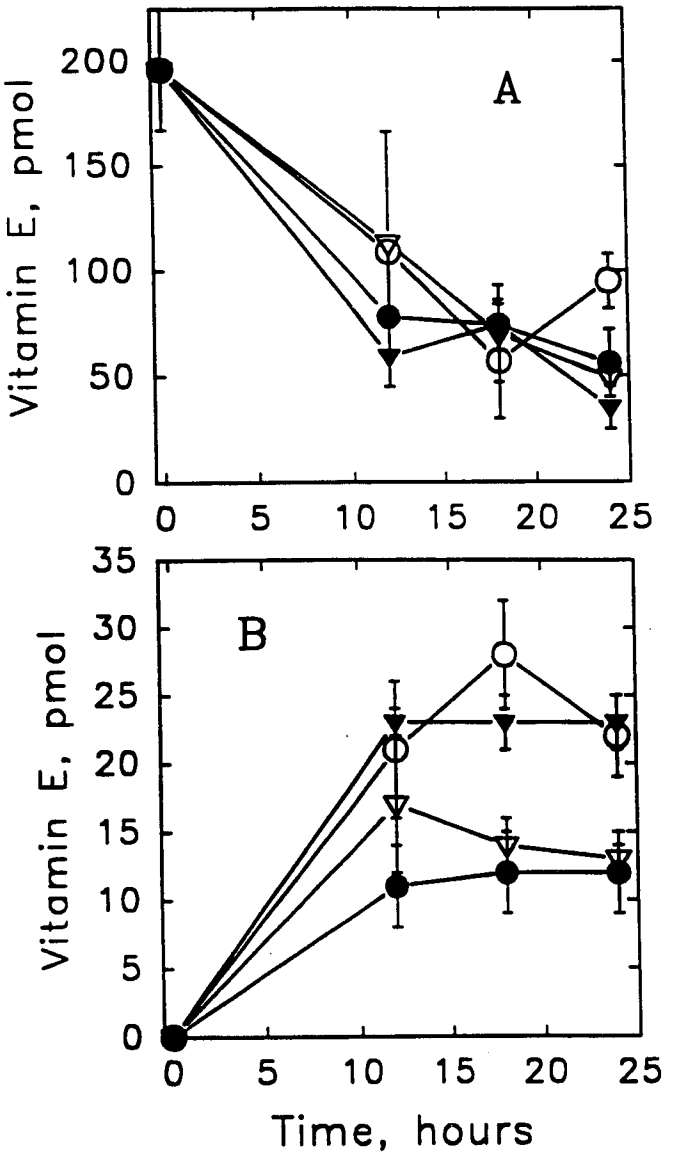


Figure 17. Effects of PMA stimulation and NO production were studied in four treatment groups of PAM in RPMI w/ 5% FBS; untreated controls (○); treated with PMA (●); treated with NMA (▾); and treated with PMA and NMA (v). Data is from 1 experiment done in triplicate, but was reproduced a second time. All values are expressed as mean ± SD.

α-TH release by cultured PAM. However, O_2^- production appears to occur in the first 3-6 hours in culture and α-TH release appears to go on for 12-24 hours. The decrease seen in iodoacetate treated cultures cannot be attributed to O_2^- production. These data argue against the theory that α-TH is oxidized in the medium at a greater rate due to stimulation of PAMs. The alternate theory that PAMs release less α-TH in these conditions needs to be investigated further.

CHAPTER 4

DISCUSSION

Vitamin E has been studied in a large assortment of model systems. This is the first detailed study of turnover of α-TH in macrophages. A notable observation of this work is the high degree of variability in initial PAM α-TH levels. This observation is consistent with those of other investigators. Mehlhorn *et al.* (1989) measured α-TH in mitochondrial membranes to study the differences in α-TH depletion kinetics between various membrane types. These authors found that α-TH levels in mitochondria varied from 34-86 ng of α-TH per mg of protein. High α-TH levels were reported in cultured endothelial cells, initially measured to be 215 ng/10^6 cells (Chan and Tran, 1990). Much lower concentrations of α-TH were observed in hepatocytes. Glascott *et al.* (1992) studied the effects of oxidative stress and α-TH in hepatocytes and reported initial concentrations between 55 and 94 ng/mg protein. Coquette *et al.* (1986) used peritoneal macrophages to determine the effect of PMA on vitamin E levels. These authors measured α-TH levels to be 260 $\pm$ 40 ng/mg protein, this level corresponds to 25 $\pm$ 2 ng/10^6 cells or approximately 240 pmol/4 x 10^6 cells. In contrast, Sakamoto *et al.* (1990) reported only 1.2 $\pm$ 0.4 ng/10^6 cells ($\approx$ 10 pmol/4 x 10^6 cells) of α-TH when looking at the relationship of α-TH and PGE_2 production in the same type of cells.

This large discrepancy in concentration of α-TH in freshly isolated macrophages can also be seen in PAM. The initial level of α-TH reported here for PAM varied from

4.3-21.5 ng/10^6 cells. This wide variation is reflected primarily by differences between platings. Figure 7A displays the combined data from 5 experiments over a 9 month period. The standard deviation at the time 0 point is quite large when compared to the time 0 deviations of individual experiments as demonstrated in figures 5A, 6A, 8A, and 11A. The initial concentrations in these experiments were as low as 45 pmol/4x10^6 cells (Figure 12A) and as high as 195 pmol/4x10^6 cells (Figure 17A). One explanation for the variation in levels of PAM vitamin E may be nutritional. There was an inverse relationship between length of time that animals were kept in the university animal care facility and the content of α-TH in the cells of those animals.

Once plated, PAM show a steady decrease in α-TH concentration for the first 12-24 hours in culture. At the end of this time, PAM lost from 60% to 80% of their initial α-TH concentration. This loss of α-TH can be the result of any combination of the turnover components, oxidation, metabolism and physical loss. Figure 10 illustrates that even non-treated macrophages can release $O_2^{\cdot-}$. Coquette *et al.* (1986) presented data that macrophages stimulated with PMA demonstrate a 40% decrease in α-TH concentration after 1 hour. Therefore, oxidation is a possible route of α-TH turnover accounting for the loss of α-TH. Apart from antioxidant reactions, little is known about cellular metabolism of α-TH, so its contribution to α-TH turnover cannot be assessed. The physical loss of α-TH can be accounted for by the secretion of α-TH or its release from dead cells.

The observed loss of α-TH is accompanied by a 12% decrease in cell viability.

The loss of α-TH began between 0-3 hours after plating cells, while cell viability was 96% at 6 hours after plating. Therefore, the loss of α-TH preceded the decrease in viability. This depletion of α-TH may depress the antioxidant capabilities of PAM predisposed to oxidative insult and may actually lead to the observed decrease in viability.

The α-TH turnover seen in the first 24 hours of culture is dominated by the release of unoxidized α-TH to the medium. Bjϕrneboe *et al.* (1987) reported the secretion of unoxidized α-TH by hepatocytes in primary culture. The authors described the release to be rapid with 50% of the α-TH secreted in the first 4 hours of culture. Their experiments linked α-TH release with VLDL secretion. By treating hepatocytes with chloroquine or colchicine, known inhibitors of VLDL secretion, α-TH secretion could be inhibited. Glascott *et al.* (1992) confirmed these results in experiments that examined effects of α-TH on oxidative stress in cultured hepatocytes. These authors reported the decrease in α-TH from 73 ng/mg protein to 12 ng/mg protein after 18-20 hours in culture. Of the α-TH lost from the hepatocytes, 40% could be accounted for in the medium as unoxidized α-TH. Both of these reports demonstrate a similar tendency of cells to release α-TH. These previous reports and our present experiments illustrate that cells can secrete or release lipophilic compounds such as α-TH into aqueous media.

PAM secretion of α-TH into the aqueous medium could only be measured in cultures grown in the presence of serum. Consequently, it was determined that α-TH had to associate with some component of the serum to be soluble in the aqueous medium.

In both of the previously cited hepatocyte experiments, cultures of hepatocytes were grown without serum supplementation of the culture medium. Since α-TH probably did not associate with any serum components, the α-TH may have been solubilized in the medium due to its incorporation into secreted lipoproteins. In previous work by Bjørneboe *et al.* (1986), it was reported that α-TH secreted by hepatocytes did not associate with serum proteins. We found that α-TH could not be detected in the medium from cultures of PAM grown in serum free Ham F12 medium supplemented with 0.5% bovine serum albumin (data not shown). These experiments show that α-TH secreted by PAMs did not associate with albumin in our system. Therefore, the component of the serum which associates with α-TH may be a lipoprotein.

Lipoproteins are synthesized in the liver and are known to transport α-TH. The incorporation of α-TH into plasma LDL *in vitro* was described by Esterbauer *et al.* (1991). A similar technique was used in this study to increase the amount of α-TH in serum exposed to PAMs. Cultures grown in the presence of serum with 250 pmol/ml medium of α-TH demonstrated a 500% increase of cellular α-TH, from 9 pmol/4 x 10^6 cells to 54 pmol/4 x 10^6 cells, over 6 hours. Chan and Tran (1990) performed similar experiments with endothelial cells cultured with α-TH enriched serum. These authors reported a 400% increase in cellular α-TH when grown in the presence of 23.2 μM α-TH. The absorbed α-TH was primarily associated with organelle membranes with concentrations highest in the plasma and mitochondrial membranes.

Further evidence of the uptake of α-TH by PAM is given in Figure 12. This

experiment was designed to determine if α-TH reuptake from the culture medium could result in reproducible increases in cellular α-TH. In all the serum supplemented cultures, cellular α-TH appeared to increase 12-18 hours after plating although the magnitude of the increase was not statistically significant. The cultures in serum free conditions showed no apparent late increase of cellular levels because of the lack of soluble α-TH in the medium (Figure 12B). However, α-TH levels in serum supplemented cultures were consistently higher than in cells in serum-free cultures. These observations collectively suggest a simple model of α-TH behavior in PAM cell culture.

The movement of α-TH in PAM culture is a dynamic process. Figure 18 is a schematic of the different interactions that have been described. The first process (1) is the release of α-TH from cultured macrophage to the aqueous medium. Once in the medium, α-TH can either associate with the surface of the culture dish (2) or with a component of the serum such as a lipoprotein (3). The α-TH which is associated with a serum component can also undergo a variety of interactions. It may be transferred to the surface of the culture dish (4), taken back up by the PAM (5) or exchanged with another component in the serum (6). Along with these physical interactions, α-TH can also undergo oxidation. With these movements of α-TH in mind, the kinetic behavior of α-TH in cell culture may be explained. Figure 19 represents a cellular concentration vs time graph typical of those from cultures in serum supplemented media. Phase I of Figure 19 represents the rapid release of α-TH by the cultured PAM. α-TH uptake by the PAM may also be occurring, but the medium concentration of α-TH is so low that

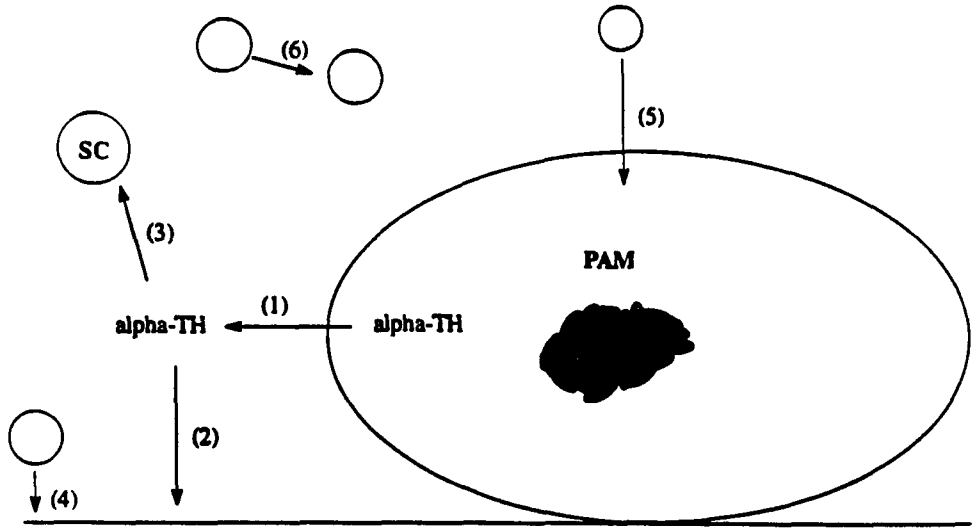


Figure 18. The movements of unoxidized α-TH in culture PAM: (1) Release of α-TH by PAM; (2) Movement from plating medium to the culture dish surface; (3) Association with components of the serum; (4) Exchange between serum components (sc) and culture plate surface; (5) Resorption by PAM; (6) Exchange between sc.

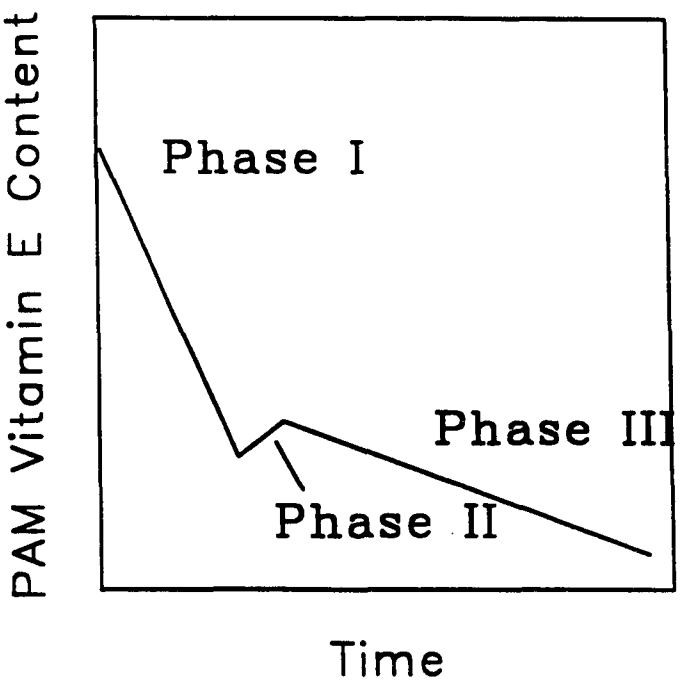


Figure 19. Schematic of PAM cellular α-TH concentration versus time.

uptake would not be detectable during the rapid α-TH release. Towards the end of phase I, cellular levels of α-TH have fallen, medium levels have risen and the release of α-TH slows. Phase II begins as the rate of α-TH release drops below that of α-TH uptake from the medium by the PAM and cellular levels begin to rise. During both phases there is exchange of α-TH occurring between the serum and plate causing an essentially irreversible removal of α-TH from this system. Additional loss can occur through oxidation. The steady loss of α-TH causes medium levels gradually to drop and the rate of α-TH uptake drops accordingly. This decrease in uptake by the macrophage leads to phase III, which shows a more gradual, steady decrease in both cellular and medium levels of α-TH. This gradual decrease reflects the removal of α-TH from the system by surface interactions and α-TH oxidation. With a theoretical model of the behavior of α-TH in culture, manipulations of oxidative stress can be performed. Future studies will be largely concerned with measuring the levels of α-TH oxidation products occurring in cell and medium samples. These studies will determine the contribution of oxidation to α-TH turnover.

A major focus of this research is to determine the effect of PAM activation upon α-TH status in culture. Coquette *et al.*(1986) examined the effect of PMA activation on the α-TH content of peritoneal macrophages. These authors found that 40% of the initial α-TH was lost 1 hour after treatment with PMA. There were no measurements of oxidation products or medium levels of α-TH. In PAM cultures treated with PMA cellular α-TH depletion was similar to that in non-treated controls (Figure 15A).

However, the amount of α-TH detected in the medium was half that found in the corresponding controls (Figure 15B). As seen in Figure 16, PAM treated with PMA will produce O_2^- for 3 to 6 hours after treatment. Therefore this decrease in α-TH detected may have been due to oxidation of α-TH. As a result, the loss of 30-40% of initial PAM to oxidation may account for the lower medium levels in cultures treated with α-TH. Cultures treated with iodoacetate demonstrate a lowering of medium α-TH similar to that seen in PMA treated cultures. Iodoacetate blocks the hexose monophosphate shunt pathway which leads to depletion of NADPH and inhibition of O_2^- production. The cytochrome-c assay demonstrated that iodoacetate treatment inhibited the production of O_2^- (Figure 16). Consequently, these results suggest that the decrease in medium α-TH levels in iodoacetate treated cultures was not related to α-TH oxidation.

This conclusion is supported further by the results of the xanthine/xanthine oxidase experiment. In this experiment, an external radical generating system was added to cultured PAM. The medium levels of α-TH in cultures treated with the pro-oxidant system and in cultures without were both identical. The presence of the radical generating system did not appear to affect the level of unoxidized α-TH in the culture medium (Figure 14B). Although the generation of radicals in these experiments was not explicitly verified, the effects of xanthine/xanthine oxidase and superoxide dismutase on cell morphology are consistent with this assumption. Two alternative hypotheses to explain the decreased levels of α-TH detected in the medium of iodoacetate-treated cultures are that NADPH depletion affected the release of α-TH by PAMs, or that

iodoacetate treatment affected α-TH release by a mechanism unrelated to NADPH status.

An additional radical which was addressed in these experiments is NO. The experimental protocol for PAM activation only exposed cells to PMA. To stimulate NO production by PAM, cells are pretreated with LPS 6-8 hours prior to stimulation (Lorsbach and Russell, 1992). Because LPS was not used in our experiments, the impact of NO upon α-TH oxidation should have been negligible. Experiments involving the use of N^G-methyl-L-arginine as an inhibitor of NO synthetase demonstrated no effect upon the behavior of α-TH in culture. These data were further supported by measurements of medium nitrite, which showed no increases in nitrite levels for any of the experimental conditions. Future experiments will have to include treatments that stimulate PAM production of NO. It would be interesting to investigate the effect of NO and $O_2^{\cdot}$ in combination on α-TH turnover, since these combine to form peroxynitrite, a precursor to very reactive radicals (Beckman *et al.*, 1990).

The release of α-TH *in vitro* has been described in a variety of cell types. Chan and Tran (1990) reported that cultured endothelial cells from human umbilical cord vein lost α-TH at a high rate for the first 15 hours in culture followed by a slower depletion over the next 3 days. Bjϕrneboe *et al.* (1987) studied the release of α-TH by cultured hepatocytes. The release by hepatocytes also was demonstrated to be initially rapid and then slow significantly. This release was linked with the secretion of VLDLs. This link correlates the *in vitro* secretion of α-TH to liver secretion *in vivo*. This raises the possibility that the release of α-TH by PAM actually represents the natural secretion of

this antioxidant *in vivo*.

The mechanisms of antioxidant delivery to the lung surfaces are not well understood. GSH is synthesized by several different cells of the lung, and PAM demonstrate the highest rate of GSH biosynthesis (Horton *et al.*, 1987). Rouzer *et al.* (1982) studied GSH turnover in peritoneal macrophages and found it to occur rapidly in these cells. These authors found that > 90% of this turnover is the result of efflux of intact GSH. This transport out of macrophages is performed by a membrane protein γ-glutamyl-transpeptidase (Meister and Anderson, 1983). These observations suggest that PAM may function to deliver antioxidants to the surface of the lung.

When stimulated by immune complexes or PMA, PAMs can synthesize and release bioactive compounds such prostaglandins, thromboxane and leukotrienes (Sibille and Reynolds, 1990). These compounds are synthesized from arachidonic acid by either cyclooxygenase or lipoxygenase and are involved in lung homeostasis. Because they are arachidonic acid metabolites, these compounds are somewhat lipophilic. The release of such compounds suggests that PAM have mechanisms to release highly lipophilic compounds. Physical acceptors for the release of lipophilic compounds by PAM may exist in the lung lining fluid. PAM do not exist in a completely aqueous environment, as pulmonary surfactant contains significant quantities of lipids (Van Golde *et al.*, 1988). The majority of lipids present are phospholipids, which may be more receptive to lipophilic molecules such as α-TH. PAM may be able to release highly lipophilic antioxidants and lipophilic components of the lung lining fluid may facilitate α-TH

release.

The possibility that PAM participate in the delivery of α-TH to the surface of the lungs could be addressed by a series of experiments. Gadolinium chloride, which inhibits PAM function (Husztik *et al.*, 1980), could be introduced by intratracheal installation to interfere with PAM activities. The broncho-alveolar lavage (BAL) fluid from these animals can be assayed for α-TH after treatment of PAM. BAL α-TH levels in GdCl treated animals can be compared to the BAL α-TH of non-treated control animals with normal populations of PAM. If normally functioning PAM are necessary for α-TH delivery, then BAL levels in GdCl-treated animals should be depressed. A second approach would be to assay the BAL fluid from an animal with a compromised immune system, such as the SCID mouse which is deficient of tissue macrophages. If PAM are involved in α-TH delivery then SCID mice should have consistently lower amounts of α-TH in their BAL fluid when compared to mice with PAM. These are simple approaches which may be able to illustrate a role for PAM in maintaining the antioxidant capabilities of the lung.

The release of α-TH by PAMs may have other consequences *in vivo*. Sharmanov *et al.* (1990) reported that α-TH has an inhibitory effect upon O_2^- production. These authors demonstrated that peritoneal macrophage deficient in α-TH had increases in oxygen radical production without increases in antioxidant enzyme activity. This led to structural and functional changes in the macrophage plasma membranes. In addition to change in radical production, α-TH deficiency led to increases in synthesis of various

bioactive lipids. Chan *et al.* (1989) found that excessive dietary vitamin E reduced PAM production of thromboxane A_2 through inhibition of phospholipase and cyclooxygenase. α-TH status may affect prostaglandin production by controlling cellular hydroperoxide tone. Sakamoto *et al.* (1991), reported a 36% decrease in PGE_2 production by peritoneal macrophages isolated from animals fed high α-TH diets. These authors report inhibition of the same enzyme systems as Chan *et al.*, however they also report inhibition of protein kinase C. These studies imply that PAM α-TH status may affect the formation of various mediatory molecules by exerting general antioxidant actions in pulmonary cells.

Vitamin E turnover in PAM may play an important role in disease and injury states of the lung. PAMs have been implicated in the mechanisms of injury associated with adult respiratory distress syndrome (ARDS), emphysema and toxicity associated with various pollutants. ARDS is believed to be caused by an exaggerated immune response triggered by sepsis (Christman *et al.*, 1991). PAM recruit neutrophils to the lung and large amounts of radical production leads to damage of the surrounding tissues. The high degree of radical production by both macrophage and neutrophils may cause depletion of cellular α-TH, which has been associated with increased radical production (Sharmanov *et al.*, 1990). This compounding relationship between α-TH depletion and radical production in PAMs may contribute to these disease states.

The study of α-TH in cultured PAMs can be applied to research of oxidative injury associated with a variety of diseases and toxicities. This project has described the

basal behavior of α-TH in cell culture. Future work is needed to characterize the oxidative turnover of α-TH in culture and its metabolic fate. Further study will be required to determine the *in vivo* significance of these *in vitro* observations. PAM cell cultures can provide a useful tool for studying the balance between oxidative stress and antioxidant protection.

LIST OF ABBREVIATIONS

α-TH	α-tocopherol, vitamin E
α-TQ	α-tocopherolquinone
AH^-	ascorbate anion
AH_2	ascorbic acid
ARDS	adult respiratory distress syndrome
BSA	bovine serum albumin
CCl_4	carbon tetrachloride
CNS	central nervous system
DNA	deoxyribonucleic acid
FBS	fetal bovine serum
γ-TH	gamma-tocopherol
GC/MS	gas chromatography/mass spectrometry
GSH	glutathione (reduced)
GSSG	glutathione (oxidized)
HOCl	hypochlorous acid
HDL	high density lipoprotein
H_2O_2	hydrogen peroxide
LDL	low density lipoprotein
LPS	lipopolysaccaride

MPO	myeloperoxidase
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NMA	N^G-methyl-L-arginine
NO	nitric oxide
O_2^-	superoxide
•OH	hydroxyl radical
$ONOO^-$	peroxynitrite
PBS	phosphate buffered saline
PAM	pulmonary alveolar macrophage
PMA	phorbol myristate acetate
SOD	superoxide dismutase
TQE	α-tocopherolquinone epoxides
VLDL	very low density lipoproteins

REFERENCES

Aruoma OI, Halliwell B, Gajewski E, Dizdarogh M (1991). Copper Ion-Dependent Damage to Bases in DNA in the Presence of Hydrogen Peroxide. Biochem. J. 273: 601-604.

Ambruso DR and Johnston RB (1981). Lactoferrin Enhances Hydroxyl Radical Production by Human Neutrophils, Neutrophil Particulate Fractions, and an Enzymatic Generating System. J. Clin. Invest. 67: 352-360.

Ashkar S, Binkley F, and Jones DP (1981). Resolution of a Renal Sulfhydrol (Glutathione) Oxidase from gamma-Glutamyl Transferase. FEBS Lett. 124: 166-168.

Babior BM, Curnotte JT, and Okamura N (1988). Blood. 72 Supplement(1): 141a

Babior BL, Rosin RE, McMurrich BJ, Peters WA, and Babior BM (1981). Arrangement of the Respiratory Burst Oxidase in the Plasma Membrane of the Neutrophil. J. Clin. Invest. 67: 1724-1728.

Beatrice MC, Palmer JW, and Pfeiffer DR (1980). The Relationship between Mitochondrial Membrane, Potential Membrane Permeability and the Retention of Ca^{++} by Mitochondria. J. Biol. Chem. 255: 8663-8671.

Beckman JS, Beckman TW, Chen J, Marshall PA, and Freeman BA (1990). Apparent Hydroxyl Radical Production by Peroxynitrite: Implications for Endothelial Injury from Nitric Oxide and Superoxide. Proc. Natl. Acad. Sci. 87: 1620-1624.

Behrens WA and Madere R (1987). Mechanisms of Absorption, Transport, and Tissue Uptake of RRR alpha-Tocopherol and d-gamma-Tocopherol. Role in the White Rat. J. Nutr. 117: 1562-1569.

Bendich A, D'Apolito P, Gabriel E, and Machlin LT (1984). Interaction of Dietary Vitamin C and Vitamin E on Guinea Pig Immune Responses to Nitrogens. J. Nutr. 114: 1588-1593.

Bendich A, Machlin JL, and Scandurra O (1986). The Antioxidant Role of Vitamin C. Free Rad. Biol. Med. 2: 419-444.

Bendich A, Machlin JL, Scandurra O, Burton GW, and Wagner DDM (1986). Adv. Free Rad. Biol. Med. 2: 419-444.

Berridge MJ (1985). The Molecular Basis of Communication within the Cell. Sci. Am. 253: 142-152.

Biemond P, Van Eijk HG, Swaak AJG, Koster JF (1984). Iron Mobilization from Ferritin by Superoxide Derived from Stimulated Polymorphonuclear Leukocytes; Possible Mechanism in Inflammation Diseases. J. Clin. Invest. 73: 1576-1579.

Bielski BH (1982). Chemistry of Ascorbic Acid Radicals. In *Ascorbic Acid: Chemistry, Metabolism and Uses*. (P.A. Seib and B.M. Tolbert, eds) 81-100, Advances in Chemistry Series: 200. Am. Chem. Soc.

Bjornson LK, Gniewkowski C, and Kayden HJ (1975). A Comparison of the Exchange of Alpha-Tocopherol and of Free Cholesterol between Rat Plasma Lipoproteins and Erythrocytes. J. Lipid Res. 16: 39-53.

Bjorneboe A, Bjorneboe GA, Hagen BF, Nossen JO, and Deven CA (1987). Secretion of Alpha-Tocopherol from Cultured Rat Hepatocytes. Biochim. Biophys. Acta. 922: 199-205.

Bjorneboe A, Bjorneboe GA, Bodd E, Hagen BF, Kuestch N, and Drevon CA (1986). Transport and Distribution of Alpha-Tocopherol in Lymph, Serum and Liver Cells in Rats. Biochim. Biophys. Acta. 889: 310-315.

Boveris A and Chance B (1973). The Mitochondrial Generation of Hydrogen Peroxide. Biochem. J. 134: 707-716.

Brown M and Goldstein JL (1986). A Receptor Mediated Pathway for Cholesterol Homeostasis. Science. 232: 34-47.

Burton GW, Daba T, Gabe EJ, Hughes L, and Lee FL (1985). Autoxidation of Biological Molecules. 4. Maximizing the Antioxidant Activity of Phenols. J. Am. Chem. Soc. 107: 7053-7065.

Burton GW and Ingold KU (1984). Beta-Carotene: An Unusual Type of Lipid Antioxidant. Science. 224: 569-573.

Burton GW and Ingold KU (1986). Vitamin E: Application of Principles of Physical Organic Chemistry to the Exploration of its Structure and Function. Accounts of Chem. Res. 19: 194.

Burton GW, Joyce A, and Ingold KU (1983). Is Vitamin E the only Lipid-Soluble, Chain Breaking Antioxidant in Human Blood Plasma and Erythrocytes? Arch. Biochem. Biophys. 221: 228-290.

Burton GW and Traher MG (1990). Vitamin E: Antioxidant Activity, Biokinetics and Bioavailabilty. Annu. Rev. Nutr. 10: 357-82.

Buttris JL and Diplock AT (1988). The Relationship between Alpha-Tocopherol and Phospholipid Fatty Acids in Rat Liver Subcellular Membrane Fractions. Biochim. Biophys. Acta. 962: 81-90.

Cadenas E, Muller A, Brigelius R, Esterbauer H, and Sies H (1983). Effects of 4-Hydroxynonenal on Isolated Hepatocytes. Biochem. J. 214: 479-487.

Chandler DB, Fuller WC, Jackson RM, and Fulmer JD (1987). Studies of Membrane Receptors and Phagocytosis in Subpopulations of Rat Alveolar Macrophages. Am. Rev. Respir. Dis. 133: 461-467.

Caren TE, Schwenke DC, and Steinberg D (1987). Antioxidants *in vivo* can Selectively Inhibit Low Density Lipoprotein Degradation in Macrophage-rich Fatty Acid Streaks and Slow the Progression of Atherosclerosis in the Watanabe Heritable Hyperlipidemic Rat. Proc. Natl. Acad. Sci. 84: 7725.

Chance B, Sies H, and Boveris A (1979). Hydroperoxide Metabolism in Mammalian Organs. Physical. Rev. 59: 527-605.

Chan AC, Tran K, Pyle D, and Powell WS (1989). Effects of Dietary Vitamin E on the Biosynthesis of 5-Lipogenase Products by Rat Polymorphonuclear Leukocytes (PMNL). Biochim. Biophys. Acta. 1005: 265-269.

Chan AC and Tran K (1990). The Uptake of (RRR) Alpha-Tocopherol by Human Endothelial Cells in Culture. Lipids. 25: 17-21.

Christman WJ, Wheeler AP, and Bernard GR (1991). Cytokines and Sepsis: What are the Therapeutic Implications? J. Crit. Care 6: 172-82.

Clack DG, McElligott TF, and Hurst FW (1966). The Toxicity of Paraquat. Br. J. Ind. Med. 23: 126-32.

Coquette A, Vray B, and Varderpus J (1986). Role of Vitamin E in the Protection of the Resident Macrophage Membrane Against Oxidative Damage. Arch. Int. Physiol. Biochem. 94: S29-S34.

Cunningham ML, Johnson JS, Giovanazzi SM, and Peak MJ (1985). Photosensitized Production of Superoxide Anion by Monochromatic (290-405) Ultraviolet Irradiation of NADH and NADPH Coenzymes. Photochem. Photobiol. 42: 125-128.

Davirskja, Delsignore ME, and Lin SW (1987). Protein damage and Degradation by Oxygen Radicals: The Modification of Amino Acids. J. Biol. Chem. 262: 9902-9907.

DeChatelot LR, Shirley PS, and Johnston RB (1976). Effect of Phorbol Myristate Acetate on the Oxidative Metabolism of Human Polymorphonuclear Leukocytes. Blood. 47: 545-554.

Dizdaroglu M (1951). Chemical Determination of Free Radical-Induced Damage to DNA. Free Rad. Bio. Med. 10: 225-242.

Edwards BA, Hallett MB, Llyod D, and Campbell AK (1983). Decrease in Apparent Km for Oxygen after Stimulation of Respiration of Rat Polymorphonuclear Leukocytes. FEBS Lett. 161: 60-64.

Esterbauer H, Dieber-Rotheneder M, Striegl G, and Waeg G (1991). Role of Vitamin E in Preventing Oxidation of Low Density Lipoproteins. Am. J. Clin. Nutr. 53: 3145-3215.

Etherington DJ, Pugh G, and Silver JA (1981). Collagen Degradation in an Experimental Inflammatory Lesion: Studies on the Role of the Macrophage. Acta. Biol. Med. Ger. 40: 1625-1636.

Fariss MW, Pascoe GA, and Reed DJ (1985). Vitamin E Reversal of the Effect of Extracellular Calcium on Chemically induced Toxicity in Hepatocytes. Science. 227: 751-754.

Fels ADS and Cohn ZA (1986). The Alveolar Macrophage. J. Appl. Physiol. 60: 353-369.

Fiedler EM, Roberts PB, Bray RC, Lowe DJ, Mautzner GN, Rotilio G, and Calabrase L (1974). Mechanism of Action of Superoxide Dismutase from Pulse Radiolysis and Electron Paramagnetic Resonance. Evidence that Only Half the Active Sites Function in Catalysis. Biochem. J. 139: 49-60.

Flohé L, Gunzler WA, and Schock (1973). Glutathione Peroxidase: A Seleno-enzyme. FEBS Lett. 32: 132-134.

Flohé L and Schlegel W (1974). Glutathione-Peroxidase, IV. Hoppe-Seyler's Z. Physiol. Chem. 352: 1401-1410.

Fridovich I (1975). Superoxide Dismutases. Annu. Rev. Biochem. 44: 147-159 (1975).

Gajewski E, Fuciarelli AF, Dizdarogiu M (1988). Structure of Hydroxyl Radical-Induced DNA-Protein Cross Links in Calf Thymus Nucleohistone *in Vitro*. Int. J. Radiat. Biol. 54: 445-459.

Getzoff ED, Taner JA, Weiner PK, Kollman PA, Richardson JS, Richardson JC (1983). Electrostatic Recognition between Superoxide and Zinc, Copper Superoxide Dismutase. Nature. 306: 287.

Glascott PA, Gilfor E, and Farber JL (1992). Effects of Vitamin E on the Killing of Cultured Hepatocytes by tert-Butyl Hydroperoxide. Mol. Pharm. 41: 1155-1162.

Granot E, Tramir I, and Deckelbaum RJ (1988). Neutral Lipid Transfer Protein Does Not Regulate Alpha-Tocopherol Transfer Between Human Plasma Lipoproteins. Lipids. 23: 17-21.

Gutteridge JMC and Halliwell B (1987). Radical-Promoting Loosely Bound Iron Free Radicals in Biological Fluids and the Bleomycin Assay. Life. Chem. Rep. 4: 113-42.

Gutteridge JMC and Toeg D (1982). Adriamycin-dependent Damage to Deoxyribose: A Reaction Involving Iron, Hydroxyl and Semiquinone. FEBS Lett. 149: 228.

Haberland ME and Fogelman AM (1987). The Role of Altered Lipoproteins in the Pathogenesis of Atherosclerosis. Am. Heart J. 113: 573-577.

Halliwell B and Gutteridge JMC (1990). Role of Free Radicals and Catalytic Metal Ions in Human Disease: An Overview. Meth. Enzymol. 186: 1-85.

Halliwell B, Gutteridge JMC, and Blake D (1985). Philos. Trans. R. Soc. London Ser. B 311: 659.

Halliwell B, Gutteridge JMC, and Cross CE (1992). Free Radicals, Antioxidants and Human Disease: Where are we now? J. Lab. Clin. Med. 119: 598-620.

Hamilton TA and Adams DO (1987). Molecular Mechanisms of Signal Transduction in Macrophages. Immunol. Today. 8: 151-158.

Hevel JM, White KA, and Marletta MA (1991). Purification of the Inducible Murine Macrophage Nitric Oxide Synthase. J. Biol. Chem. 266: 22789-22791.

Hershko C, Graham G, Bates GW, and Rachmilewitz EA (1978). Non-Specific Serum Iron in Thalassemia: An Abnormal Serum Iron Fraction of Potential Toxicity. Br. J. Haematol. 40: 255.

Hildebrandt AG and Roots I (1975). Reduced Nicotinamide Adenine Dinucleotide Phosphate (NADPH)-Dependent Formation and Breakdown of Hydrogen Peroxide During Mixed Function Oxidation Reactions in Liver Microsomes. Arch. Biochem. Biophys. 171: 385-397.

Handelman GJ, VanKuijk FJGM, Chatterjee A, and Krinsky NI (1991). Characterization of Products Formed During the Autoxidation of Beta-Carotene. Free Rad. Biol. Med. 10: 427-437.

Hogg JC (1982). Neutrophil Kinetics and Lung Injury. Physiol. Rev. 67: 1249-1293.

Horton JK, Meredith MJ, and Bend JR (1987). Glutathione Biosynthesis from Sulfur-Containing Amino Acids in Enriched Populations of Clara and Type II Cells and Macrophages Freshly Isolated from Rabbit Lung. J. Pharm. Exp. Therap. 240: 376-380.

Howard JA and Ingold KU (1967). Absolute Rate Constants for Hydrocarbon Autoxidation. VI. Alkyl Aromatic and Olefinic Hydrocarbons. Can. J. Chem. 45: 793-802.

Husztik E, Lazar G, and Parducz A (1980). Electron Microscopic Study of Kupffer-Cell Phagocytosis Blockade Induced by Gadolinium Chloride. Br. J. Exp. Path. 61: 624-630.

Iyengar R, Stuehr DJ, and Marletta MA (1987). Macrophage Synthesis of Nitrite, Nitrate, and N-nitrosamines: Precursors and Role of the Respiratory Burst. Proc. Natl. Acad. Sci. 84: 6368-6373.

Joester KE, Jung G, Weber U, and Weser U (1972). Superoxide Dismutase Acitivity of Cu 2+ -Amino Acid Chelates. FEBS Lett. 25: 25-28.

Kagan VE, Bakalova RA, Serbinova EA, and Stojtcher TS (1990). Fluorescence Measurement of Incorporation and Hydrolysis of Tocopherol and Tocopherol Esters in Biomembranes. Meth. Enzymol. 186: 355-367.

Kamp DW, Dunne M, Weiteman SA, and Dunn MM (1989). The Interaction of Asbestos and Neutrophils Injures Cultured Human Pulmonary Epithelial Cells: Role of Hydrogen Peroxide. J. Clin. Med. 114: 604-612.

Knowles RG, Palacios M, Palmes RMJ, and Moncada S (1989). Formation of Nitric Oxide from L-Arginine in the Central Nervous System: a Transduction Mechanism for Stimulation of the Soluble Guanylate Cyclase. Proc. Natl. Acad. Sci. 86: 5159-5162.

Laroff GP, Fossender RW, and Schuler RH (1972). The Electron Spin Resonance Spectra of Radical Intermediates in the Oxidation of Ascorbic Acid and Related Substances. J. Am. Chem. Soc. 94: 9062-9073.

Lash LH and Jones DP (1982). Localization of the Membrane-Associated Thiol Oxidase of Rat Kidney to Basal-Lateral Plasma Membrane. Biochem. J. 203: 371-378.

Levine RL (1983). Oxidative Modification of Glutamine Synthetase. J. Bioc. Chem. 258: 1123-1127.

Leung KP and Goren MB (1989). Uptake and Utilization of Human Polymorphonuclear Granule Myeloperoxidase by Mouse Peritoneal Macrophages. Cell Tissue Res. 257: 653-656.

Liebler DC, Baker PF, and Kaysen KL (1990). Oxidation of Vitamin E: Evidence for Competing Autoxidation and Peroxyl Radical Trapping Reactions of the Tocopheroxyl Radical. J. Am. Chem. Soc. 112: 6995-7000.

Liebler DC and Burr JA (1992). Oxidation of Vitamin E During Iron-Catalyzed Lipid Peroxidation: Evidence for Electron-Transfer Reactions of the Tocopheroxyl Radical. Biochem. 31: 8278-8284.

Lorsbach RB and Russell SW (1992). A specific Sequence of Stimulation is Required to Induce Synthesis of the Antimicrobial Molecule Nitric Oxide by Mouse Macrophages. Infect. Immun. 60: 2133-2135.

Machlin LJ (1991). Vitamin E. In *Handbook of Vitamins: 2nd Edition.* Machlin LJ (ed.). Marcel Dekker, New York, 99-144.

Mahoney LR (1970). Evidence for the Formation of Hydroxyl Radicals in the Isomerization of Pernitrous Acid to Nitric Acid in Aqueous Solution. J. Am. Chem. Soc. 92: 5262-5263.

McKay MJ and Bond JS (1985). In *Intracellular Protein Catabolism.* Alan R. Liss, New York, 351.

McRipley RJ and Sharra AJ (1967). Role of the Phagocyte in Host-Parasite Interactions. XII. Hydrogen Peroxide-Myeloperoxidase Bactericidal System in the Phagocyte. J. Bacteriol. 94: 1425-1430.

Mehlhorn RJ, Sumida S, and Packer L (1989). Tocopheroxyl Radical Persistence and Tocopherol Consumption in Liposomes and in Vitamin E - Enriched Rat Liver Mitochondria and Microsomes. J. Biol. Chem. 284: 13448-13452.

Meinster A and Anderson ME (1983). Glutathione. Ann. Rev. Biochem. 52: 711-760.

Moser U and Bendich A (1991). In *Handbook of Vitamins: 2nd edition.* Machlin LJ (ed.). Marcel Dekker, New York, 195-232.

Mowri H, Nakagawa Y, Inoue K, and Nojima (1981). Evr. J. Biochem. 117: 537.

Murphy DJ and Mavis RD (1981). Membrane Transfer of Alpha-Tocopherol. J. Biol. Chem. 256: 10464-10468.

Osaki S, Johnson DA, and Frieden E (1989). The Possible Significance of Ferrous Oxidase Activity of Ceruloplasmin in Normal Human Serum. J. Biol. Chem. 241: 2746.

Pacht ER, Timerman AP, Lykens MG, and Merola AJ (1991). Deficiency of Alveolar Fluid Glutathione in Respiratory Distress Syndrome. Chest 100: 1397-1403.

Packer JE, Slater TF, and Wilson RL (1979). Direct Observation of a Free Radical Interaction Between Vitamin E and Vitamin C. Nature. 278: 737-738.

Pacifici RE and Davies KJA (1990). Protein Degradation as an Index of Oxidative Stress. Meth. Enzymol. 186: 485-495.

Pascoe GA, Fariss MW, Olafsdottir K, and Reed DJ (1987). Role of Vitamin E in Protection Against Cell Injury. Maintenance of Intra-cellular Glutathione Precursors and Biosynthesis. Eur. J. Biochem. 166: 241-247.

Prutz WA, Butler J, and Land EJ (1983). Int. J. Radiat. Res. 16: 483-502.

Prutz WA, Monig H, Butler J, and Land EJ (1985). Reactions of Nitrogen Dioxide in Aqueous Model Systems: Oxidation of Tyrosine Units in Peptides and Proteins. Arch. Biochem. Biophys. 243: 125-134.

Pryor WA (1991). The Antioxidant Nutrients and Disease Prevention - What do we know and what do we need to find out? Am. J. Clin. Nutr. 53: 3915-3935.

Pryor WA (1986). Oxy-Radicals and Related Species: Their Formation, Lifetimes and Reactions. Ann. Rev. Physiol. 48: 652-67.

Pryor WA, Strickland T, and Church DF (1988). A Comparison of Efficiencies of Several Natural and Synthetic Antioxidants in Aqueous Sodium Dodecyl Sulfate Micelle Solutions. J. Am. Chem. Soc. 110: 2224-2229.

Rabani J, Klug-Roth D, and Lilie J (1973). Pulse Radiolytic Investigations of the Catalyzed Disproportionation of Peroxy Radicals: Aqueous Cupric Ions. J. Phys. Chem. 77: 1169-1175.

Reynolds HY, Atkinson JP, Newball HA, and Frank MM (1975). Receptors for Immunoglobulin and Complement on Human Alveolar Macrophages. J. Immunol. 114: 1813-1819.

Richter C, and Kass (1991). Gen. Oxidative Stress in Mitochondria: It's Relationship to Cellular Ca^{++} Homeostasis, Cell Death, Proliferation and Differentiation. Chem. Biol. Interactions. 83: 1-23 (1991).

Rotilio G, Bray RC, and Fielden EM (1972). A Pulse Radiolysis Study of Superoxide Dismutase. Biochim. Biophys. Acta. 268: 605-609.

Rouzer CA, Scott WA, Griffith OW, Hamilton AL, and Cohn ZA (1982). Glutathione Metabolism in Resting and Phagocytizing Peritoneal Macrophages. J. Biol. Chem. 257: 2002-2008.

Rowley DA and Halliwell B (1982b). Superoxide-Dependent Formation of Hydroxyl Radicals from NADH and NADPH in the Presence of Iron Salts. FEBS Lett. 142: 39-41.

Rowley DA and Halliwell B (1982a). Superoxide-Dependent Formation of Hydroxyl Radicals in the Presence of Thiol Compounds. FEBS Lett. 138: 33-36.

Scarpa M, Rigo A, Maiorino M, Ursiani F, and Gregolin C (1984). Formation of Alpha-Tocopherol Radical and Recycling of Alpha-Tocopherol by Ascorbate During Peroxidation of Phosphatidylcholine Liposomes. An Electron Paramagnetic Resonance Study. Biochim. Biophys. Acta. 801: 215-219.

Sibille and Reynold (1990). Macrophage and Polymorphonuclear Neutrophils in Lung Defense and Injury. Am. Rev. Respir. Dis. 141: 471-501.

Sokol RJ (1988). Vitamin E Deficiency and Neurologic Disease. Ann. Rev. Nutr. 8: 351-373.

Strauss RR, Paul BB, Jacobs AA, and Sharra AJ (1970). Role of the Phagocyte in Hostiparasite Interactions: XXII. Hydrogen Peroxide-dependent Decarboxylation and Deamination by Myeloperoxidase and its Relationship to Antimicrobial Activity. J. Reticuloendothel. Soc. 7: 754-761.

Stuehr DJ and Marletta MA (1987). Synthesis of Nitrate and Nitrite in Murine Macrophage Cell Lines. Cancer Res. 47: 5590-5594.

Traber MG and Kayden HJ (1984). Vitamin E is Delivered to Cells via the High Affinity Receptor for Low Density Lipoproteins. Am. J. Clin. Nutr. 40: 747-751.

Traber MG and Kayden HJ (1989). Preferential Incorporation at Alpha-Tocopherol vs Gamma-Tocopherol in Human Lipoproteins. Am. J. Clin. Nutr. 49: 517-526.

Traber MG, Olivecrona T, and Kayden HJ (1985). Bovine Milk Lipoprotein Lipase Transfers Tocopherol to Human Fibroblasts During Triglyceride Hydrolysis *in Vitro*. J. Clin. Invest. 75: 1729-1734.

von Golde, Batenburg, and Robertson (1988). Pulmonary Surfactant System. Physiol. Rev. 68: 360-445.

von Oud Ablas Bard and Furth R (1979). Origin, Kinetics, and Characteristics of Pulmonary Macrophages in the Normal Steady State. J. Exp. Med. 149: 1504-1518.

von Sonntag C (1987). The Chemical Basis of Radiation Biology. London: Taylor & Francis.

Weiland JE, Davis WB, Holter JF, Mohammed JR, Dornsky PM, and Gadek JE (1986). Lung Neutrophils in the Adult Respiratory Distress Syndrome: Clinical and Pathophysiology Significance. Am. Rev. Respir. Dis. 133: 218-225.

Wendel A, Pilz RW, Landenstein R, Sawatzki G, and Weset U (1975). Substance-induced Redox Change of Selenium in Glutathione Peroxidase Studied by X-Ray Photoelectron Spectroscopy. Biochim. Biophys. Acta. 377: 211-215.

Weib Z, Foler R, and Munck A (1978). Interaction of Glucocorticoids with Macrophages. Identification of Glucocorticoid Receptors on Monocytes and Macrophages. J. Exp. Med. 147: 1684-1694.

White R, Janoff A, and Godfrey JP (1980). Secretion of Alpha-2-Macro-globulin by Human Alveolar Macrophage. Lung. 158: 9-14.

Winkler P, Linder W, Esterbauer H, Schauenstein E, Schaur RJ, and Khoschsorur GA (1984). Detection of 4-Hydroxynonenal as a Product of Lipid Peroxidation in Native Ehrlich Acites Tumor Cells. Biochim. Biophys. Acta. 796: 232-236.

Winterle J, Dulin D, and Mill T (1984). Products and Stoichiometry of Reaction of Vitamin E with Alkylperoxy Radicals. J. Org. Chem. 49: 491-495.

Yamauchi R, Matsui T, Satake Y, Kato K, and Ueno Y (1989). Reaction Products of Alpha-Tocopherol with a Free Radical Initiator, 2,2'-Azo*bis*(2,4-dimethylvaleronitrile). Lipids. 24: 204-209.