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**RIBOFLAVIN PHOTSENSITIZED SINGLET OXYGEN OXIDATION
OF VITAMIN D**

DISSERTATION

**Presented in Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy in the Graduate School of
The Ohio State University**

By

Joan M. King, M.S.

*** * * * ***

The Ohio State University

1996

**Dissertation Committee
David B. Min
Grady Chism
John Litchfield
Matrid Ndife**

Approved by

A handwritten signature in black ink, appearing to read "D. B. Min", is written over a horizontal line.

**Adviser
Food Science and Nutrition**

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ABSTRACT

The riboflavin photosensitized singlet oxygen oxidation of vitamin D₂ in a model system of 12% water and 88% acetone was studied. Samples containing 0, 3000, 6000 or 9000 ppm vitamin D₂ and 0, 5, 10, or 15 ppm riboflavin and 0, 50 or 60 ppm ascorbic acid and 0, 40 and 60 ppm α -tocopherol were prepared in triplicate. Sixteen ml of each sample was transferred into a 25 ml serum bottle. The sample bottle was sealed air-tight with a Teflon-lined rubber septum and an aluminum cap. Samples bottles were stored in a 4,000 lux light storage box or in the dark at 45°C for 1, 2, 4 and 8 hours. The oxidation of vitamin D₂ in the sample was determined by measuring the headspace oxygen depletion in the bottle during storage. The headspace oxygen was determined by injecting 100 microliters of headspace gas into a thermal conductivity gas chromatograph.

Vitamin D₂ in the samples without riboflavin and stored in the light or vitamin D₂ in the samples with riboflavin and stored in the dark was not oxidized during the storage period.

However, vitamin D₂ in samples with riboflavin and stored in the light was oxidized. The loss of headspace oxygen in the samples ranged from 9.56% to 57.3% during eight hours of storage under light depending on the concentration of riboflavin and vitamin D₂. The higher the riboflavin or vitamin D₂ concentration or the longer the storage period under light, the greater was the oxidation of vitamin D₂. The results indicate that vitamin D₂ is oxidized by riboflavin-photosensitized singlet oxygen.

The oxidized vitamin D₂ product was separated by HPLC and analyzed by UV, FTIR and MS spectroscopy. Only samples which had been stored in the light and contained both vitamin D₂ and riboflavin showed a new peak during HPLC separation. Analysis of the new peak by UV, MS and FTIR confirmed that it was an oxidation product of vitamin D₂. FTIR analysis showed that the oxidation product was different from untreated vitamin D₂. MS analysis showed an increase in the molecular weight of 16, the exact weight of oxygen. The absorbance maximum for the oxidation product was 250 nm while that for untreated vitamin D₂ was 264 nm. This meant that vitamin D₂ had been oxidized at the triene section of its structure. Further analysis of the MS spectrums revealed that the oxidized vitamin D₂ product was most likely an epoxide at position 5,6.

Dedicated to all of the students currently A.B.D.

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VITA

July 5, 1962..... Born - Waterville, Maine

1985..... B.S. in Chemistry
University of Maine
Orono, Maine

1989..... M.S. in Food Science
University of Maine
Orono, Maine

1989-1993..... Research Associate
Opta Food Ingredients
Bedford, Massachusetts

1993-1996..... Graduate Research Associate
Department of Food Science
and Technology
The Ohio State University
Columbus, Ohio

PUBLICATIONS

King, J.M. and Min, D.B. Fatty Acids in Analyzing Food for Nutritional Labeling and Hazardous Contaminants, Marcel Dekker, New York, NY, 1994.

King, J.M., McEvily, A.J. and Iyengar, R. Liquid Chromatographic Determination of the Processing Aid 4-Hexylresorcinol in Shrimp. *Journal of the Association of Official Analytical Chemists*. 74:1003. 1991.

King, J.M. Chemical and Physical Changes in Blueberries Associated with Maturation, Preprocess Lag Time, Processing and Storage Conditions and the Partial Characterization of Blueberry Pectin. M.S. Thesis. University of Maine. 1989.

Bushway, R.J., Pask, W.M., King, J., Perkins, B. and Ferguson, B.S. Determination of Chloradane in Soil by Enzyme Immunoassay. *First International Symposium: Field Screening Methods for Hazardous Waste Site Investigations*. Printed by Life Systems, Inc. 1988.

Bushway, R.J., King, J.M., Perkins, B. and Krishnan, M. High Performance Liquid Chromatographic Determination of Ascorbic Acid in Fruits, Vegetables and Juices. *Journal of Liquid Chromatography*. 11:3415. 1988.

Bushway, R.J., Perkins, L.B. and King, J.M. Simultaneous Determination of Diazinon and Chlorpyrifos Pesticide Formulations by Liquid Chromatography. *Journal of the Association of Official Analytical Chemists*. 71:321. 1988.

Bushway, R.J., Bureau, J.L. and King, J. Modification of the Rapid High Performance Liquid Chromatographic Method for the Determination of Potato Glycoalkaloids. *Journal of Agricultural and Food Chemistry*. 34:277. 1986.

FIELDS OF STUDY

Major Field: Food Science

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INTRODUCTION

Vitamin D is important for normal mineralization and growth of bones. It acts as a prohormone which when hydrolyzed to 1,25-dihydroxyvitamin D, stimulates absorption of calcium in the intestine. There are two main forms of vitamin D, cholecalciferol, D₃ and ergocalciferol, D₂ (Parish, 1979). Vitamin D₃ is formed through synthesis in animal by sunlight conversion of 7-dehydrocholesterol in the skin. Vitamin D₂ is formed in plants by sunlight conversion of ergosterol. Both are used in the fortification of milk and infant formulas to help prevent rickets in children and osteomalacia in adults.

On the other hand, drinking milk with excess vitamin D can cause hypervitaminosis (Jacobus et al., 1992 and Takeuchi et al., 1989). Hypervitaminosis causes hypercalcemia, kidney stones, extraskeletal calcification and oxidation of tissue lipids. Increased levels of free radicals were observed by electron spin resonance spectroscopy (ESR) in animal tissues after dosage with excess levels of vitamin D (Spirichev and Sergeev,

1988). Intake of antioxidants such as tocopherol reduced peroxidation and the formation of kidney stones.

Several studies have been done to determine the effects and metabolic fate of vitamin D during supplementation. Takeuchi et al. (1989) found higher plasma levels of 25-hydroxyvitamin D₂ in the breast milk of lactating mothers who had received a dosage of 1200 IU/day vitamin D₂. More vitamin D₂ than 25-hydroxyvitamin D₂ was found in the breast milk, but the level of 1,25-dihydroxyvitamin D₂ decreased with time of lactation. Only a slight increase in vitamin D activity was observed.

Reddy and Tserng (1990) studied the metabolic fate of 25-hydroxyvitamin D₂ and found that it was converted to 24,25-dihydroxyvitamin D₂ and two new metabolites 24,25,28-trihydroxyvitamin D₂ and 24,25,26-trihydroxyvitamin D₂. Significant amounts of the later two metabolites were found in the plasma of rats induced with hypervitaminosis D₂. It was hypothesized that the formation of these metabolites plays an important role in the deactivation of 25-hydroxyvitamin D₂ which would be important in prevention of calcification of soft tissues caused by hypervitaminosis D₂.

Vitamin D can undergo oxidation itself, due to its conjugated triene structure. This fact has been utilized to study the metabolism of vitamin D. The biological activity of various derivatives formed from the oxidation of vitamin D by

singlet oxygen was determined (Moriuchi et al., 1979). Rose Bengal was used as a sensitizer for the formation of singlet oxygen. Two isomeric peroxides were formed, but only one was as active as vitamin D₃, (6R)-6,19-epidloxy-9,10-seco-5(10),7-cholestadien-3B-ol.

As far as stability in milk goes, most studies only analyzed photooxidation of vitamins A, C and riboflavin (Dimmick, 1982 and Sattar and deMan, 1975). Renken and Warthesen (1993) found that vitamin D was lost in a model acetonitrile system or in skim milk after storage with oxygen present. Light did not affect vitamin D loss in acetonitrile, but did cause loss of vitamin D in skim milk. The authors hypothesized that the loss of vitamin D was due to photosensitized oxidation by singlet oxygen, but did not test this hypothesis.

Since sensitizers are required for the formation of singlet oxygen, and riboflavin, which is in milk, is a known sensitizer, my first objective was to prove the hypothesis that riboflavin photosensitized singlet oxygen oxidation of vitamin D does occur. My second objective was to determine the kinetics and quenching mechanisms of singlet oxygen oxidation of vitamin D in order to retard or prevent singlet oxygen oxidation of vitamin D. Because it is known that vitamin D degrades in the presence of light and oxygen and various metabolites and products of vitamin D may interfere with calcium metabolism,

my third objective was to identify the main oxidation product of vitamin D.

LITERATURE REVIEW

1. Vitamin D Structure and Nutritional Significance

The two main forms of vitamin D are cholecalciferol (D_3) and ergocalciferol (D_2). Vitamin D_2 is formed in plants from the precursor ergosterol, while vitamin D_3 is formed in the skin from 7-dehydrocholesterol (Figure 1,2, Parish, 1979).

Figure 1. Formation of ergocalciferol from ergosterol by irradiation with ultraviolet light

Figure 2. Formation of cholecalciferol from 7-dehydrocholesterol by irradiation with ultraviolet light

Both are formed when the precursors are exposed to sunlight and both follow the same metabolic pathway. Ingested vitamin D which is absorbed by the lymphatic system and vitamin D produced in the skin are both transported in protein bound form by the circulatory system to the liver. Vitamin D is transformed to 25-hydroxylate vitamin D which is transported again by the circulatory system to the kidney, where 1,25-dihydroxy vitamin D is formed (Figure 3, Kreutler and Czajka-Narins, 1987). 1,25-Dihydroxy vitamin D is the active form of vitamin D which functions like a hormone to regulate dietary calcium absorption.

Vitamin D deficiency causes rickets in children, which is caused by inadequate mineralization in bones during growth and causes osteomalacia in adults, which is caused by demineralization

Figure 3. Metabolic functions of vitamin D

of fully developed bones. Excess vitamin D can cause hypervitaminosis which results in over calcification of soft tissues such as the heart, kidney and lungs. Hypervitaminosis without medical treatment can result in death.

The recommended daily requirement for vitamin D is 400 IU for children and adults (Miller and Norman, 1984). There was debate on whether this level may be too high for adults, since vitamin D can be produced by exposure to sunlight.

2. Vitamin D Sources and Stability

Although milk is fortified with vitamin D at the 400 IU level, there are also natural food sources of vitamin D such as herring and fish-liver oils which contain 333 IU per 100 g and up to 10,000 IU per 100 g, respectively (Table 1, Miller and Norman, 1984).

Table 1. Food sources of vitamin D

<u>Food</u>	<u>I.U./100 g</u>
cod-liver oil	10,000
herring	333
pork	40
cheese	12
milk	4
spinach	0.2

Vitamin D is stable to heat, acid and alkali but is unstable when exposed to light and can undergo oxidation in air after 72 hours. Renken and Warthesen (1993) found that vitamin D was lost in a model acetonitrile system or in skim milk after storage with oxygen present. Light did not affect vitamin D loss in acetonitrile, but did cause loss of vitamin D in skim milk.

3. The Chemistry of Oxygen

There are two main forms of oxygen, triplet and singlet. Figure 4 shows the molecular orbital structure of triplet oxygen (Korycka-Dahl and Richardson, 1978). There are two antibonding electrons of the same spin direction residing in separate orbitals. Each electron has a quantum spin number of

Figure 4. Molecular orbital structure of triplet oxygen

+1/2 with a total spin of 1, so the spin multiplicity is $2S+1$ which equals 3, hence the name triplet oxygen. Triplet oxygen follows Hund's principle of maximum multiplicity which states that single electrons must occupy all orbitals at the same energy level before pairing occurs. This means that triplet oxygen is a diradical compound and can react only with radical compounds. In autooxidation of lipids a radical compound is formed during the initiation step where a hydrogen is abstracted from an unsaturated fatty acid such as linoleic acid. The linoleic acid is then susceptible to oxidation by triplet oxygen.

Figure 5 shows the orbital structure of singlet oxygen which also has two antibonding electrons, but this time the two electrons are paired in the same orbital with opposite spins of +1/2 and -1/2 for a total spin of zero (Korycka-Dahl and Richardson, 1978). This results in a spin multiplicity of $2(0)+1=1$ and this is why it is referred to as singlet oxygen. Singlet oxygen does not follow Hund's principle of maximum multiplicity and is not a radical compound. Because it is not a radical compound and since it has an electron-free orbital, singlet oxygen likes to react with electron rich, non-radical compounds. In this case, unsaturated fatty acids can react directly with singlet oxygen without an initiation step. Singlet oxygen exists at an energy state of 22.4 kcal higher than triplet oxygen and is

Figure 5. Molecular orbital structure of singlet oxygen

known to react 1400 times faster than triplet oxygen with linoleic acid (Figure 6, Rawls and Van Santen, 1970).

Figure 6. Reaction of singlet oxygen vs. triplet oxygen with linoleic acid

4. Singlet Oxygen Oxidation

4.1 Sensitizers

There are four main requirements for singlet oxygen oxidation to occur. There must be a sensitizer, light, oxygen and a substrate. There several sources of sensitizers, chlorophylls from plants, riboflavin in milk, protoporphyrins in meat and synthetic colorants like Red No. 3. Sensitizers have unsaturated ring structures which can easily absorb UV light (Figure 7).

Figure 7. Structure of chlorophyll

When the sensitizer absorbs UV light it is transformed from its ground state into an excited singlet state which then becomes a triplet excited state through intersystem crossing. Once in the triplet state, the sensitizer can react with triplet oxygen to produce singlet oxygen which then reacts with an electron rich, non-radical substrate.

4.2 Quenching Mechanisms and Kinetics

Quenchers typically have highly conjugated unsaturated structures, such as α -tocopherol, dimethylfuran and carotenoids like β -carotene (Figure 8).

Figure 8. Structure of β -carotene

All of the possible reactions during singlet oxygen oxidation shown in Figure 9. There are two main points of attack in the

Figure 9. Reactions involved in singlet oxygen oxidation

prevention of singlet oxygen oxidation by the quenching mechanism. A quencher may interact with either the triplet sensitizer or singlet oxygen. When the quencher interacts with the triplet sensitizer, the sensitizer returns to its ground electronic state and no longer can react with triplet oxygen to form singlet oxygen.

Once singlet oxygen is formed by triplet sensitizer, there are three ways its reaction with substrate can be prevented (Figure 9). One way is through decay in a solvent. Singlet oxygen has a short lifetime and its duration depends on the type of solvent in the system. Singlet oxygen's stability ranges from 3 to 5 μsec in water to 240 to 300 μsec in chloroform.

Singlet oxygen also may chemically react with the quencher to oxidize it or may physically react with the quencher to form triplet oxygen.

The type of quenching can be discerned by determining the steady state kinetics of the mechanisms (Foote, 1979). Equation (1) defines mechanisms involved in singlet oxygen oxidation:

Equation 1

$$d[AO_2]/dt = K \{ ko[O_2]/(kQ[Q] + ko[O_2]) \} \{ kr[A]/(kr[A] + kq[Q] + kox-Q[Q] + kd) \}$$

a reciprocal of which will result in Equation (2):

Equation 2

$$\{d[AO_2]/dt\}^{-1} = K^{-1} \{ (ko[O_2] + kQ[Q])/ko[O_2] \} \{ [kr[A] + (kox-Q + kq)[Q] + kd]/kr[A] \}$$

where AO_2 , A and Q are the concentrations of the oxidized substrate, the substrate and the quencher, respectively, K^{-1} is the rate of formation of singlet oxygen, Ko is the reaction rate constant of triplet sensitizer with triplet oxygen, kr is the

reaction rate constant of compound with singlet oxygen, k_q is the reaction rate constant of physical quenching of singlet oxygen, K_{ox-Q} is the reaction rate constant of chemical quenching of singlet oxygen and k_d is the decay rate of singlet oxygen in a solvent.

There is a linear relationship between the reciprocal of the formation rate of oxidized compound and the reciprocal of the concentration of substrate. Quenching mechanisms and rates can be determined by graphing the reciprocals for each level of quencher used. If oxidation is being prevented by the interaction of quencher with triplet sensitizer then equation (2) becomes:

Equation 3

$$\{d[AO_2]/dt\}^{-1} = K^{-1} \{1 + (k_Q[Q]/k_o[O_2])\} \{1 + (k_d/kr[A])\}$$

A graph of the reciprocals of $[AO_2]$ vs. $[A]$ at various quencher levels, $[Q]$, shows different intercepts, which is indicative of triplet sensitizer quenching (Figure 10). The ratio, of the slopes of these plots to their intercepts, where the slope = $K^{-1} \{k_d(k_Q[Q] + k_o[O_2])/k_o[O_2]kr\}$, the intercept = $K^{-1} \{(k_o[O_2] + k_Q[Q])/k_o[O_2]kr\}$ and slope/intercept = $\{k_d(k_Q[Q] + k_o[O_2])/k_o[O_2]kr\} \{k_o[O_2]/(k_o[O_2] + k_Q[Q])\} = k_d/kr$, is independent of quencher concentration.

When singlet oxygen is being quenched, Equation (2) becomes:

Equation 4

$$\{d[AO_2]/dt\}^{-1} = K^{-1} \{1 + (k_{ox} - Q + k_q)[Q] + k_d\} / k_r[A]$$

Figure 10. Steady state kinetics graph of triplet sensitizer quenching

The plots of the reciprocals of $[AO_2]$ as a function of $[A]$ at various $[Q]$ show different slopes where slope = $K^{-1} \{k_d + (k_{ox} - Q + k_q)[Q]\} / k_r$, but a constant intercept, K^{-1} (Figure 11). The ratio of the slopes to the intercept is dependent on quencher concentration. The ratio of slope/intercept = $\{k_d + (k_{ox} - Q + k_q)[Q]\} / k_r$ and a plot of

slope/intercept as a function of $[Q]$ results in a line with an intercept $= k_d/k_r$ and a slope $= (k_q + k_{ox}\cdot Q)/k_r$ (Figure 12).

Figure 11. Steady state kinetics graph of singlet oxygen quenching

Figure 12. Graph of the ratios of slopes/Intercepts of the regression lines of Figure 11 as a function of quencher concentration.

4.3 General Reaction Types of Singlet Oxygen

There are several reaction types of singlet oxygen, 1,4 addition to heterocyclic compounds, 1,2 cycloaddition to olefins, oxidation of sulfides, oxidation of electron rich aromatic compounds, oxidation of phenols and ene reactions with olefins (Wasserman and Murray, 1979).

4.3.1 1,4 Addition to Heterocyclic Compounds

Heterocyclic systems include those of furans, pyrroles, indoles, imidazoles, purines, oxazoles, thiazoles and thiophenes (Wasserman, H.H. and Lipshutz, B.H., 1979). Ketones are the main products resulting from singlet oxygen reaction with heterocyclic systems. Prior to ketone formation, peroxides are formed across the oxygen atom in furans, across the amine constituent in pyrroles and across the sulfur constituent in thiazoles and thiophenes (Figures 13, 14, 15, 16).

Figure 13. Oxidation of furan

Figure 14. Oxidation of 2,3,4,5-tetraphenyl-pyrrole

Figure 15. Oxidation of 2,4,5-triphenylthiazole

Figure 16. Oxidation of 1,3-diphenylisobenzothiophene

Singlet oxygen can attack indoles at the double bond at position 2 on the amine ring and purines are attacked at substituted amine positions or at the double bond between the two rings (Figures 17, 18).

Figure 17. Oxidation of methylindole

Figure 18. Oxidation of xanthine

Imidazoles can be attacked across the amine constituent, while oxazoles are attacked across the oxygen atom and both can be attacked across the double bond at position 4 (Figures 19, 20, 21).

Figure 19. Oxidation of di-substituted imidazole

Figure 20. Oxidation of tri-substituted imidazole

Figure 21. Oxidation of tri-substituted oxazole

4.3.2 1,2 Cycloaddition to Olefins

1,2 Cycloaddition reactions occur at double bonds in olefins (Schaap, P. and Zaklika, K.A., 1979). The intermediate product may be a dioxetane or hydroperoxide, either of which result in ketone or aldehyde end products following cleavage of the double bond (Figures 22, 23).

Figure 22. Oxidation of Olefin

Figure 23. Oxidation of dihydropyran

4.3.3 Oxidation of Sulfides

Sulfides react with singlet oxygen to form an intermediate persulfoxide before the final sulfoxide product (Figure 24, Foote, 1976).

Figure 24. Oxidation of dialkyl sulfide

4.3.4 Oxidation of Electron Rich Aromatic Compounds

These compounds react with singlet oxygen to produce a peroxide intermediate formed across the ring structure (Salto and Matsuura, 1979). The peroxide then converts to an epoxide which can further be reacted to form a ketone (Figure 25).

Figure 25. Oxidation of 1,2,4,5-tetramethoxybenzene

Peroxides can also form at double bonds connecting two ring structures which will split to form two ketones (Figure 26).

Figure 26. Oxidation of 9,9'-bifluorenylidene

4.3.5 Oxidation of Phenols

Oxidation of phenols by singlet oxygen results in the formation of a ketone where the hydroxy group once was (Foote, 1976). Hydroperoxides can also form due to the double bonds of the ring (Figure 27, 28).

Figure 27. Oxidation of 2,3,6-trimethylphenol

Figure 28. Oxidation of 2,6-di-tert-butylphenol

4.3.6 Ene Reactions with Olefins

Both hydroperoxides and dioxetanes can be formed by singlet oxygen ene reactions with olefins (Gollnick and Kuhn, 1979). The type of reaction depends on whether there is substitution on the ring (Figure 29, 30).

Figure 29. Oxidation of 1-methyl-4a,5,6,7,8,8a-trans-hexahydronaphthalene

Figure 30. Oxidation of 1,4a-dimethyl-5,6,7,8,8a-trans-hexahydronaphthalene

Allylic compounds can form hydroperoxides which will react further with singlet oxygen to produce peroxides by 1,4 cycloaddition (Figure 31).

Figure 31. Oxidation of β -myrcene

5. Oxidation in Food

The main food constituents that are susceptible to singlet oxygen oxidation in foods are proteins, lipids and vitamins. Other constituents such as flavor compounds can also be attacked by singlet oxygen.

5.1 Oxidation of Proteins

The aromatic and sulfur containing amino acids in proteins can react with singlet oxygen (Foote, 1976). Methionine reacts to form a sulfoxide and cysteine and cystine can also be oxidized by singlet oxygen (Figure 32).

Figure 32. Oxidation of methionine

Skim milk exposed to sunlight or fluorescent light developed an sulfurous off-flavor after 30 minutes of storage (Jung et al, 1994). Samples with methionine or cyteine and riboflavin as a sensitizer were placed in light storage to test the theory that these amino acids reacted with singlet oxygen to produce the sulfurous off-flavor. Sensory evaluation and GC/MS analysis showed that methionine produced dimethyl disulfide in the presence of riboflavin, light and oxygen possibly by the following reaction:

Tryptophan has been shown to react with singlet oxygen to form N-formylkynurenine which can react as a sensitizer to form more singlet oxygen (Figure 33, Foote, 1976). This reaction may

Figure 33. Oxidation of tryptophan

proceed by a 1,2 cycloaddition across the double bond of the nitrogen ring to form a 1,2 dioxetane prior to the ketone and aldehyde groups. Histidine reacts with singlet oxygen to form aspartic acid by addition of singlet oxygen across the double bond nearest to the carbonyl to form a dioxetane or endoperoxide (Figure 34, Foote, 1976).

Figure 34. Oxidation of histidine

5.2 Oxidation of Lipids

Autooxidation of fatty acids requires the initiation process where a metal can abstract a hydrogen from the bond requiring the least energy to break (Figure 35). Linoleic acid can first be attacked at position 11 between the two double bonds at which the energy required for breaking the bond is about 50 kcal (Labuza, 1971). It can also be attacked at positions 8 and 14 at which the energy required is 70 kcal. Oxidation proceeds further with a reorganization to a

conjugated double bond system followed by propagation of peroxides by reaction of di-radical triplet oxygen with the radical on the lipid. The end result is a termination step where

Figure 35. Autooxidation of linoleic acid

the chain breaks apart to form volatile non-radical compounds that cause off-flavor.

Singlet oxygen oxidation of lipids does not require an initiation step since both the lipid and singlet oxygen are non-radical compounds. The singlet oxygen directly attacks the double bonds of the lipids and can form both conjugated and non-conjugated hydroperoxides. Autooxidation by triplet oxygen can only form conjugated hydroperoxides. Ultraviolet spectroscopy of the hydroperoxides can help to distinguish between singlet and triplet oxygen oxidation (Rawls and Van Santen, 1970).

Cholesterol is another lipid fraction substrate which can be oxidized by singlet oxygen (Korycka-Dahl and Richardson, 1978). The main product, 3 β -hydroxy-5 α -hydroperoxy-d Δ^6 -cholestene, is a carcinogen which can cause free radical oxidation of fatty acids after breaking down (Figure 36).

Figure 36. Oxidation of cholesterol

5.3 Oxidation of Flavor Compounds

Limonene is the major flavor component in orange juice and is known to react with singlet oxygen as shown in Figure (37, Gollnick and Kuhn, 1979).

Figure 37. Oxidation of (+)-limonene

Just as with lipids, different products are formed when limonene reacts with triplet or singlet oxygen (Figure 38). α -Terpinene which is found in orange oil will react with singlet oxygen to form ascaridole (Figure 39, Korycka-Dahl and Richardson, 1978).

Figure 38. Gas chromatographic analysis of limonene autooxidation and singlet oxygen oxidation products

Figure 39. Oxidation of α -terpinene

The flavor compounds in beer hops, humulones and lupulones (Figure 40), oxidize with singlet oxygen to form 3-methyl-2-butene-1-thiol which causes sunlight off-flavor (Rosenthal, 1985). Hexahydrocolupulone and tetrahydrohumulone, the acid compounds which give beer a bitter flavor can be peroxisomally oxidized by singlet oxygen to give an stronger bitter flavor to beer.

Figure 40. Structures of flavor compounds in beer hops

5.4 Oxidation of Synthetic Antioxidants

Synthetic antioxidants have many double bonds which makes them certain targets for oxidation by singlet oxygen (Foote, 1976). Butylated hydroxytoluene follows the general reaction of phenols which results in the formation of a compound with a ketone substituent in place of the hydroxyl group and a hydroperoxide on the opposite side of the ring (Figure 41).

Figure 41. Oxidation of butylated hydroxytoluene

5.5 Oxidation of Vitamins

5.5.1 Riboflavin

Riboflavin is a 7,8-dimethyl-10-(1'-D-ribityl) isoalloxazine (Figure 42) which can enter into the body from foods in three forms: flavin adenine dinucleotide (FAD), riboflavin 5'-phosphate (FMN) and free riboflavin (Cooperman and Lopez, 1984). Riboflavin must be hydrolyzed to the free form before it can be absorbed into the small intestine mucosal cells. After phosphorylation to FMN, the riboflavin is attached to plasma albumin in the portal system and transported to the liver to be converted to FAD (Cooperman and Lopez, 1984). FAD is mainly stored in the liver, kidney and heart. FAD and FMN function as oxidases or dehydrogenases to facilitate

electron transfer in oxidation-reduction reactions in biological systems. FAD is involved with fatty acid oxidation and the

Figure 42. Structure of riboflavin

Kreb's cycle as a coenzyme in the synthesis of ATP (Cooperman and Lopez, 1984). A deficiency in riboflavin will affect the metabolism of glucose, fatty acids and amino acids, but an excess of riboflavin in the diet showed no toxic effects.

The recommended daily allowance (RDA) for riboflavin is 0.6 mg (Cooperman and Lopez, 1984). Riboflavin is found in highest amounts in milk and meat ranging from 0.17 mg/100g in milk to 3.5 mg/100g in liver. Riboflavin is fairly stable to heat processing, but is very labile when exposed to light such as in milk stored in glass bottles. The main product in the photodegradation of riboflavin under acidic or neutral conditions is lumichrome which formed in milk exposed to

either sunlight or fluorescent light (Parks and Allen, 1977 and Allen and Parks, 1979). Lumiflavin is formed under alkaline conditions through the loss of the ribityl side chain (Palanuk and Warthesen, 1988). Riboflavin photodegraded by first order kinetics at a reaction rate of $1.5 \times 10^{-5} \text{ s}^{-1}$ in whole milk and $1.9 \times 10^{-5} \text{ s}^{-1}$ in skim milk when exposed to fluorescent light at 2690 lux (Allen and Parks, 1979). There was a 50% loss of riboflavin in dry, uncooked macaroni exposed to a light intensity of 150 ft-c for 3 hours, followed by a gradual linear decrease (Furuya and Warthesen, 1984). Only riboflavin in the surface of the macaroni can interact with light. This observation may explain this rapid decrease followed by a gradual one. The original riboflavin level had little effect on the percentage of riboflavin lost by photodegradation which averaged 60% after two days of storage and 70% after seven days of storage of five samples (Furuya and Warthesen, 1984).

Several studies were done to determine the factors affecting the stability of riboflavin. Riboflavin dissolved in water was found to be stable after 4 hours of storage at 50°C and at acidic pH, but not under basic conditions above pH 7 (Kearsley and Rodriguez, 1981). Riboflavin was less stable at a temperature of 75°C or higher. An increase in water activity, controlled by adding glycerol to an aqueous solution, caused a increase in riboflavin photodegradation, as did light exposure, even at a pH of 2.3. Ascorbic acid alone or glycine with

tryptophan added to a model food system stored at 79°C helped stabilize riboflavin (Kearsley and Rodriguez, 1981). An increase from 0.11 to 0.75 in the water activity of macaroni caused an increase in the amount of photodegradation of riboflavin (Furuya et al., 1984). There was also an increase in riboflavin degradation with an increase in light intensity of storage from dim light to 100 ft-c. The rate constants for the degradation of riboflavin were higher for liquid samples than for dry samples with non-fat dry milk having the lowest rate constant of -0.274 day^{-1} and phosphate solution having the highest at -2.016 day^{-1} (Furuya et al., 1984). The increase in the concentration of lumichrome during photodegradation of riboflavin was not high enough to account for the total loss of riboflavin. Less than 30% of the riboflavin lost was accounted for (Furuya et al., 1984). Another study found that only 23% of riboflavin in skim milk, exposed to 1614 lux fluorescent light for up to 120 hours, was oxidized to lumichrome (Palanuk and Warthesen, 1988).

The effects of fat, solids and light intensity levels and agitation, sampling location, coverage with films and processing on the stability of riboflavin in milk samples were determined (Gaylord et al., 1986 and Palanuk et al., 1988). Increases in the fat content of milk resulted in lower photodegradative losses of riboflavin, ranging from 70% loss for skim milk to 55% loss for whole milk (Gaylord et al., 1986). Slightly less riboflavin was lost with the addition of non-fat dry milk with rate constants of

0.0281 h⁻¹ for 0% to 0.0251 h⁻¹ for 1%. Both added solids and added fat result in less light penetration into the milk, so less riboflavin is lost (Gaylord et al., 1986). More agitation of milk samples and increased light intensity up to 300 ft-c result in higher losses of riboflavin (Gaylord et al., 1986 and Palanuk et al., 1988). Milk samples that were not agitated had a difference in riboflavin concentration with sampling location. Riboflavin levels in milk were higher at the bottom of the polyethylene containers than at the top which was closer to the light source (Palanuk et al., 1988). There was less photodegradation of riboflavin if test tubes containing milk were covered with films of low transparency (Palanuk et al., 1988). Cooking and canning caused the destruction of riboflavin in shrimp with losses of 32% and 67%, respectively (Gordon et al., 1979). Freezing did not affect the stability of riboflavin in various types of seafood. There was no loss of riboflavin in wheat, maize, mungbeans and chickpeas treated with cobalt-60 gamma irradiation up to 5 kGy (Khattak and Klopfenstein, 1989).

Riboflavin stability was also tested in the form of a mononucleotide, riboflavin 5'-phosphate (FMN), which was made into a aluminum lake (Huang and Byerly, 1992). FMN lake and FMN alone were made into pastes with Avicel and exposed to fluorescent light of 100 or 210 ft-c. The water activity was kept to 0.44 or 0.32. FMN lake was always more stable than FMN in light storage. The FMN photodegraded

rapidly, similar to riboflavin in the macaroni (Furuya and Warthesen, 1984), in the first 12 hours of storage followed by a linear degradation, but the FMN lake showed a gradual decrease throughout 3 days of storage (Huang and Byerly, 1992). The rate constant for photodegradation of FMN at a water activity of 0.44 and storage under 100 ft-c light was 2.39 days^{-1} , while that for FMN lake was 0.19 days^{-1} under the same conditions.

In summary, riboflavin is less stable under basic conditions, at temperatures greater than 75°C , with increases in water activity, increases in light intensity during storage, increased agitation of the sample, decreased distance from the light during storage and under cooking and canning conditions. Riboflavin is more stable under acidic conditions, in samples of higher fat content, higher solids content, under frozen conditions, in samples protected from light, during Cobalt 60 gamma irradiation and as an FMN aluminum lake.

Riboflavin not only photodegrades itself, but also causes the oxidation of various substrates such as malondialdehyde (MDA), tryptophan and ascorbic acid. Samples containing 0.05 M phosphate buffer, $75 \mu\text{M}$ nitro blue tetrazolium, $2 \mu\text{M}$ riboflavin and various concentrations of MDA alone or with $10 \mu\text{M}$ methionine were stored under eight fluorescent lamps in transparent tubes for ten minutes (Du and Bramlage, 1993). As the concentration of MDA increased more superoxide ion was produced which reduced the nitro blue tetrazolium as measured

by an increase in the absorbance at 560 nm. The MDA was oxidized as an electron donor and methionine slightly increased the amount of superoxide ion produced (Du and Bramlage, 1993).

The photooxidation of tryptophan in the presence of riboflavin was also studied (Kanner and Fennema, 1987). Samples of 1×10^{-3} M tryptophan and 2×10^{-5} M riboflavin were prepared at various pHs and stored under two 40 W light bulbs with an intensity of 470 ft-c. Oxidation of tryptophan increased with increased temperature from 8°C to 20°C and did not occur at low pH of 4 or 5.6 to the same extent as pH 7.5 (Kanner and Fennema, 1987). The addition of EDTA, sodium azide or ascorbic acid at pH 7.5 resulted in less oxidation of tryptophan, with ascorbic acid working the best in this system to slow oxidation. None of the antioxidants prevented oxidation completely.

Riboflavin can also cause photosensitized oxidation of ascorbic acid. Samples were prepared in citric acid buffer and saturated with oxygen for 15 minutes before being exposed to a 100 W tungsten filamented lamp at 25°C (Sahbaz and Somer, 1993). Ascorbic acid concentrations of the samples were analyzed every 5 or 10 minutes of which the initial concentration was 1.14×10^{-3} M. The rate of oxidation of ascorbic acid increased with increased riboflavin concentration and pH. The rate of oxidation ranged from 0.3×10^6 M min⁻¹ for 3.2×10^{-7} M riboflavin at pH 2.5 to 92.6×10^6 M min⁻¹ for $3.2 \times$

10^{-5} M riboflavin at pH 6.0 (Sahbaz and Somer, 1993). The rate of ascorbic acid oxidation at pH 6.0 with no riboflavin present was 4×10^6 M min⁻¹ while that with 3.2×10^{-5} M riboflavin at pH 6.0 under anearobic conditions was 13.2×10^6 M min⁻¹. The anearobic oxidation of ascorbic acid was attributed to the formation of hydrogen peroxide (Sahbaz and Somer, 1993).

Jung et al. (1995) also found that an increase in riboflavin concentration from 0 to 6 ppm caused increased oxidation from an original concentration of 1.2×10^{-4} M ascorbic acid. An increase in light intensity from 1100 lux to 5500 lux also caused an increase in the oxidation of ascorbic acid at a riboflavin level of 1.2 ppm (Jung et al., 1995). Riboflavin was 1.4x and 21x more active than methylene blue or protoporphyrin, respectively, in photosensitizing the oxidation of ascorbic acid.

5.5.2 Ascorbic Acid

Ascorbic acid is an L-threo-hex-2-enonic acid g-lactone (Figure 43) and is absorbed into the body through the intestine with the help of sodium ions (Jaffe, 1984). It is distributed widely within the body with the highest amounts located in the pituitary, adrenal, thymus and retina. L-Ascorbic acid functions as a cofactor for hydroxylating enzymes and also as an antioxidant (Jaffe, 1984). In carnitine and collagen synthesis, L-

ascorbic acid protects the hydroxylase enzymes by oxidizing ferrous ions. The metabolism of L-tyrosine requires ascorbic acid as a cofactor for the enzyme p- hydroxyphenolpyruvate

Figure 43. Structure of ascorbic acid

oxidase and the metabolism of histamine is facilitated by the presence of L-ascorbic acid and copper ions. L-ascorbic acid also enhances the adsorption of iron (Jaffe, 1984). Deficiencies in ascorbic acid results in mild symptoms such as fatigue to severe symptoms of scurvy which can result in death. Excess ascorbic acid is excreted in the urine or in unmetabolised form in the feces, so no toxic effects are possible.

The recommended daily requirement for ascorbic acid is 60 mg/day for adults and 45 mg/day for children (Jaffe, 1984). The best sources of ascorbic acid are fruits and vegetables, ranging from 200 mg/100g in peppers to 1300 mg/100g in

West Indian cherries. Ascorbic acid is a very labile vitamin, more so than riboflavin with several factors, besides exposure to light, affecting its stability. Its main oxidation product is dehydroascorbic acid.

Many studies were done on the stability of ascorbic acid in juice. Saguy et al. (1979) found that the degradation of ascorbic acid in concentrated grapefruit juice was mainly due to anaerobic degradation. Higher temperatures up to 90°C and higher solids concentration up to 60° Brix caused an increase in loss of ascorbic acid during thermal processing. The degradation of ascorbic acid in orange juice in TetraBrik cartons during storage was found to proceed both by anaerobic and aerobic reaction (Kennedy et al., 1992). This time degradation was due mainly to the aerobic process and the anaerobic reaction became a factor after the concentration of dissolved oxygen leveled off. The loss of dissolved oxygen correlated with the degradation of ascorbic acid and both were lost more rapidly at higher temperatures (Kennedy et al., 1992). Forty percent of the ascorbic acid was lost after 64 days of storage at 4°C, while 98% was lost after only 6 days of storage at 76°C. The overall rate of loss of ascorbic acid at 37°C was $3.75 \text{ mg liter}^{-1} \text{ day}^{-1}$ with an initial rate of loss of $18.3 \text{ mg liter}^{-1} \text{ day}^{-1}$ for the first 3 days (Kennedy et al., 1992). Orange juice serum, clarified of solids, had similar rates of degradation as orange juice with increasing temperature from 70.3°C to 97.6°C under anaerobic

conditions (Johnson et al., 1995). The rate constants for nonenzymatic browning of orange juice and orange juice serum were greater than those for ascorbic acid degradation and all of the rate constants were larger with increasing °Brix at each temperature. The rate constant for ascorbic acid degradation under anearobic conditions ranged from $0.43 \times 10^{-4} \text{ min}^{-1}$ for 12.7 °Brix and 70.3°C to $80 \times 10^{-4} \text{ min}^{-1}$ for 80.6 °Brix and 97.6°C (Johnson et al., 1995). The rate constant for nonenzymatic browning ranged from $10.5 \times 10^{-4} \text{ min}^{-1}$ to $347.1 \times 10^{-4} \text{ min}^{-1}$ for the same levels of temperature and °Brix.

The stability of ascorbic acid in milk and a dehydrated food system has also been tested. Pasteurization at 74°C for 16 seconds degraded 8%, 17% and 46% of the ascorbic acid in whole, 1% and chocolate milk, respectively. The difference between samples fortified with ascorbic acid, vitamin A and vitamin D followed by pasteurization and samples fortified without pasteurization were 6%, 12% and 17% ascorbic acid for whole, 1% and chocolate milk, respectively (Head and Hansen, 1979). The greatest overall loss of ascorbic acid after storage of all three samples for 36 days at 40°F was 53% for fortified 1% milk, but the whole and chocolate milks fortified with vitamins D and A before pasteurization and stored for 36 days had losses of 63% ascorbic acid. The initial ascorbic acid content of chocolate milk was 1.44 mg in 100 g while that of whole and 1% milk were 2.24 mg in 100 g and 2.36 mg in 100 g, respectively

(Head and Hansen, 1979). The lower concentration of ascorbic acid in the chocolate milk was thought to have been caused by reaction of ascorbic acid with copper ion which is found in Dutch processed cocoa.

Studies on the effect of sucrose or citric acid on the oxidation of ascorbic acid also showed that metal ions were a factor in the degradation of ascorbic acid. Ascorbic acid in aqueous solutions oxidized more quickly at higher pHs and temperatures (Hsieh and Harris, 1993). The rate constant for the oxidation of ascorbic acid in water at pH 3 and 30°C was $6.33 \times 10^{-4} \text{ min}^{-1}$ while that at pH 5 and 30°C was $69.25 \times 10^{-4} \text{ min}^{-1}$. At pH 4.25, the rate constant rose from $23.36 \times 10^{-4} \text{ min}^{-1}$ to $48.42 \times 10^{-4} \text{ min}^{-1}$ with a temperature increase from 26.5°C to 33°C (Hsieh and Harris, 1993). The addition of 10% sucrose resulted in a decrease in the rate constant to $34.17 \times 10^{-4} \text{ min}^{-1}$ for pH 4.2 and 33°C, but at lower pHs the rate constant increased when sucrose was added and this was thought to be caused by the metal impurities in sucrose. The effect of sucrose in the higher pH buffered systems was thought to be due to physical retardation of oxygen moving through the system (Hsieh and Harris, 1993).

In a solution of ascorbic acid with added citric acid and 7.9×10^{-6} to $7.9 \times 10^{-5} \text{ M Cu(II)}$, the rate constants for oxidation of the ascorbic acid increased with increasing pH from 2.5 to 4.5, but decreased from 4.5 to 6.0 (Sahbaz and Somer, 1993). This

was due to the complexation of Cu(II) with citric acid (CI) to form the most stable complex of ClCu^{2-} . Complexes formed at pHs other than 6.0 were less stable so the copper was more available to catalyze the oxidation of ascorbic acid (Sahbaz and Somer, 1993).

Rate constants for the degradation of ascorbic acid in fortified dehydrated model food systems, packaged in thermal death time cans, increased with increases in water activity in storage at 30°C (Dennison et al., 1977). The type of packaging also affected the stability of ascorbic acid due to the differences in oxygen permeability. The ascorbic acid in dehydrated product at a water activity of 0.4 was most stable in thermal death time cans with a rate constant of $3.13 \times 10^{-2} \text{ days}^{-1}$ and the least stable in 303 enameled cans which had a rate constant of $8.56 \times 10^{-2} \text{ days}^{-1}$. This was due to the fact that the 303 cans had a large amount of oxygen in the can, while the thermal death time cans were filled so that no headspace remained (Kirk et al., 1977).

Orange juice stored in clear glass bottles for five weeks after hot filling lost more ascorbic acid to oxidation than juice stored in cans for five weeks, 61% and 38%, respectively (Maeda and Mussa, 1986). All samples were stored on the laboratory bench at room temperature. Light became a factor in the degradation of ascorbic acid in the glass bottles. The addition of sodium metabisulfite or cysteine helped stabilize the ascorbic

acid in juice stored in glass bottles. Only 38% of the ascorbic acid was lost in five weeks of storage with 400 ppm of sodium metabisulfite present and the addition of 200 ppm or 400 ppm cysteine gave results similar to canned orange juice with losses of 39% and 40% ascorbic acid, respectively (Maeda and Mussa, 1986). The authors noted, but did not explain the fact that the rate of oxidation of ascorbic acid was highest for the sample containing 400 ppm cysteine, 2.87 mg per 100 ml. This may be due to the fact that cysteine itself is susceptible to oxidation. It may have accelerated the oxidation of ascorbic acid even though the concentration of ascorbic acid after 5 weeks was similar to the sample containing 200 ppm cysteine of which the rate of oxidation of ascorbic acid was 1.79 mg per 100 ml (Maeda and Mussa, 1986). Naim et al. (1993) also found that the level of ascorbic acid remaining in orange juice, stored for 14 days in glass vials at 45°C, was higher for samples containing from 2.5 mM to 9.2 mM cysteine than samples without cysteine.

Jung et al. (1995) tested several amino acids and their effect on ascorbic acid oxidation photosensitized by riboflavin and methylene blue. Cysteine, phenylalanine and alanine all inhibited oxidation of ascorbic acid, while histidine and tyrosine caused greater oxidation than what occurred in samples without amino acid present. Tryptophan decreased oxidation for the first 3 minutes, but increased oxidation after 6 minutes of storage with riboflavin under light (Jung et al., 1995). When methylene

blue was used as the sensitizer, all of the amino acids except cysteine promoted oxidation of ascorbic acid.

In summary, ascorbic acid is less stable with small increases in temperature, increases in °Brix, increases in Cu^{+2} , increases in pH, increases in light intensity, and the addition of histidine, tyrosine or tryptophan. Ascorbic acid is more stable in samples with added cysteine, sodium metabisulfite, phenylalanine or alanine.

Ascorbic acid can cause the oxidation of other substrates in a similar manner to riboflavin. After 330 minutes of storage in vials at 30°C, 45% of the methanethiol was oxidized to dimethyltrisulfide when 450 ppm ascorbic acid and 1 ppm hydrogen sulfide were present (Chin and Lindsay, 1994). If 4 ppm Fe(III) was also present, then 70% of the methanethiol oxidized to dimethyltrisulfide. Fe(III) alone with hydrogen sulfide caused only 10% of the methanethiol to oxidize to dimethyltrisulfide.

Ascorbic acid can also retard the oxidation of other substrates. Ascorbic acid is known to be a quencher of singlet oxygen and its effect in preventing oxidation in skim milk in the presence of riboflavin has been studied (Jung et al., 1994). Dimethyl disulfide was shown to be a factor in the off-flavor of light exposed skim milk. Ascorbic acid at 500 or 1000 ppm significantly ($p < 0.05$) decreased dimethyl disulfide formation in skim milk exposed to fluorescent light at 2400 lux for 6 or 8

hours. The dimethyl disulfide may be formed by singlet oxygen reacting with methionine at the sulfur group. The ascorbic acid reacts with the singlet oxygen before singlet oxygen can react with methionine. All of the samples containing ascorbic acid were given better flavor scores than control samples without ascorbic acid.

Yang (1994) showed that ascorbic acid reacted with singlet oxygen at a rate of $3.08 \times 10^8 \text{ M}^{-1} \text{ sec}^{-1}$ in an aqueous solution buffered at pH 7 stored for 1 hour under a 200 watt light bulb. Singlet oxygen was produced by photosensitization with 40 ppm Red No. 3. Higher levels of ascorbic acid resulted in increased loss of headspace oxygen with and without Red No. 3 present, but Red No. 3 caused a 3.3 fold increase overall in the loss of headspace oxygen.

5.5.3 Tocopherol

There are two main forms of vitamin E, tocopherols and tocotrienols, which make up 8 compounds that have vitamin E activity. The most active form of vitamin E is α -tocopherol (Figure 44). α -Tocopherol is absorbed in the small intestine as a lipid-bile micelle, is transported to the liver by the portal vein and is bound to lipoproteins which are found in the lymph and blood (Machlin, 1984). α -Tocopherol is stored mainly in the adipose

tissue, liver and muscle and was proposed to act as an *in vivo* antioxidant because it is located in the membranes of cells where oxygen-containing free radicals are produced. Vitamin E deficiency can cause problems ranging from hemolytic anemia in low birth weight

Figure 44. Structure of α -tocopherol

infants to neuromuscular deficits in adults, but vitamin E is relatively non-toxic when given in high doses causing minor side effects such as fatigue dermatitis and gastrointestinal symptoms (Machlin, 1984).

The recommended daily allowance of vitamin E ranges from 4.5 IU/day for infants to 15 IU/day for adults males. Vitamin E is found in highest amounts in vegetable oils and nuts ranging from 79 $\mu\text{g/g}$ in soybean oil to 1194 $\mu\text{g/g}$ in wheat germ oil, with almonds containing 270 $\mu\text{g/g}$ (Machlin, 1984).

Vitamin E as tocopherol, is very stable to heat and alkali without oxygen present and is also stable to acid. Tocopherols are very unstable in the presence of light and oxygen and oxidation results in the formation of tocopheroxide, tocopheryl quinone and tocopheryl hydroquinone (Machlin, 1984).

The effects of added chlorophylls, β -carotene and other natural and synthetic antioxidants on photooxidation of soybean oil were analyzed (Vianni, 1980). Higher levels of chlorophyll than those found at natural levels were required to accelerate oxidation of soybean oil. Low levels of β -carotene showed a noninhibiting effect instead of singlet oxygen quenching in the presence of chlorophyll and higher levels caused an increase in oxidation rates. Both chlorophyll and β -carotene degraded in samples stored under UV light and in non-irradiated samples. Without chlorophyll present, α - and γ -tocopherol did not inhibit oxidation under UV light, but prevented photooxidation under incandescent and fluorescent light. The UV light was thought to produce singlet oxygen which then degraded the tocopherols. The tocopherols may have protected β -carotene in the presence of chlorophyll, so they could act as singlet oxygen quenchers. UV radiation promoted oxidation more than fluorescent and incandescent light.

The interaction effects of three levels of chlorophyll and four levels each of β -carotene and tocopherol on the

photosensitized oxidation of soybean oil was studied (Choe and Min, 1992). Tocopherol did not have much effect on volatile formation without chlorophyll or β -carotene present. Fewer volatiles were formed when both tocopherol and β -carotene were present and this was probably due to the light filtering effects of β -carotene. When chlorophyll was present in the samples, 20 ppm of β -carotene and 1,000 ppm of tocopherol worked best at decreasing the oxidation of soybean oil and acted synergistically at the 8 ppm level of chlorophyll to prevent oxidation greater than either used alone. This was thought to be due to tocopherol protecting β -carotene from degradation.

MATERIALS AND METHODS

1. Materials

Analytical reagent grade acetone (Mallinckrodt Specialty Chemical Co., Paris, KY) was used for sample preparation. Ergocalciferol (vitamin D₂), riboflavin, α -tocopherol and ascorbic acid were obtained from Sigma Chemical Company (St. Louis, MO). HPLC grade acetonitrile and methanol (Fisher Scientific) and helium and oxygen were obtained from the chemical store at The Ohio State University.

2. Determination of Riboflavin-Photosensitized Singlet Oxygen Oxidation of Vitamin D₂

Samples were prepared by dissolving 0, 5, 10 and 15 ppm riboflavin in 12% distilled water/88% acetone solutions in separate aluminum foil-covered 200 ml volumetric flasks. Sixteen milliliters of each riboflavin solution was added to 25 ml serum bottles containing 0, 3000, 6000 or 9000 ppm of vitamin D₂. The bottles were capped air-tight with Teflon-

faced rubber septa and aluminum seals (Supelco, Inc., Bellefonte, PA). Samples were prepared in triplicate, covered with aluminum foil and shaken until the vitamin D₂ was completely dissolved. The aluminum foil was removed prior to storage.

Two sets of samples were stored at 45°C, one set in the dark as a control and one set in a mirrored wooden box (70 cm x 50 cm x 60 cm) lighted with a 200 watt tungsten bulb (GE, Cleveland, OH) at an intensity of 4,000 lumens. The samples were placed on a motor (Talboys Engineering Co., Emerson, NJ) driven rotating plastic disk (60 rpm) for uniform light exposure (10). The headspace of each sample was analyzed every 1, 2, 4 and 8 hours for oxygen content after equilibration to 20°C in a water bath for 10 to 15 minutes.

3. Sample Preparation for the Analysis of the Kinetics and Quenching Mechanisms of α -Tocopherol and Ascorbic acid on Riboflavin-Photosensitized Singlet Oxygen Oxidation of Vitamin D₂

Samples were prepared by dissolving 15 ppm riboflavin and 0, 40 or 60 ppm α -tocopherol or 0, 50 or 60 ppm ascorbic acid in 12% distilled water/88% acetone solutions in separate aluminum-foil covered 200 ml volumetric flasks. Sixteen milliliters of each riboflavin solution was added to 25 ml serum

bottles containing 0, 3000, 6000 or 9000 ppm of vitamin D₂. The bottles were capped air-tight with Teflon-faced rubber septa and aluminum seals (Supelco, Inc., Bellefonte, PA). Samples were prepared in triplicate, covered with aluminum foil and shaken until the vitamin D₂ was completely dissolved. The aluminum foil was removed prior to storage.

Samples were stored at 45°C in a mirrored wooden box (70 cm x 50 cm x 60 cm) lighted with a 200 watt tungsten bulb (GE, Cleveland, OH) at an intensity of 4,000 lumens (Yang, 1994). The samples were placed on the same motor driven rotating plastic disk used previously (60 rpm) for uniform light exposure (10). The headspace of each sample was analyzed after 8 hours for oxygen content after equilibration to 20°C in a water bath for 10 to 15 minutes.

4. Headspace Oxygen Analysis

Gas chromatographic headspace analysis was used to determine the extent of vitamin D₂ oxidation which occurred in the samples (10). A Hewlett Packard 5890 (Avondale, PA) gas chromatograph with thermal conductivity detection and a stainless steel molecular sieve column (13X, 80/100, Alltech, Deerfield, IL) was utilized. High purity helium gas (99.995%) at a flow rate of 30 ml/min was used as the carrier and auxiliary gas. The injector, detector and oven temperatures were 120°C,

150°C and 40°C respectively. One hundred microliters of headspace gas was injected into the column. Laboratory air was used as a daily reference to correct for chromatographic variation. A Hewlett Packard HP3390A (Avondale, PA) integrator was used to record the peaks in electronic counts. Statistical analysis was performed by analysis of variance using the general linear model procedure of the Statistical Analysis System (SAS) system at The Ohio State University.

5. Sample Preparation for the Separation of Vitamin D₂ Oxidation Products

Samples were prepared by placing 3000 ppm Vitamin D₂ into each of four 25 ml serum bottles, with two containing sixteen milliliters of 12% water/88% acetone only and the other two containing sixteen milliliters of 15 ppm riboflavin in 12% water/ 88% acetone. One of each sample set was placed in the dark and one in the light at 45°C for 8 hours.

6. Separation of Vitamin D₂ Oxidation Products

High performance liquid chromatography (HPLC) was used to separate the vitamin D₂ oxidation products. A Hewlett Packard 1050 HPLC (Avondale, PA) with ultraviolet detection at 254 nm and a Econosphere C₁₈ column (Alltech, Deerfield, IL)

was utilized. Helium was used to sparge the mobile phase prior to starting the pumping system. Methanol and acetonitrile were used in a gradient system to facilitate separation and ensure that less polar constituents were eluted completely. The gradient was 50/50 acetonitrile/methanol for 15 minutes followed by a linear increase to 75/25 acetonitrile/methanol over 10 minutes, holding at 75/25 for 1 minute and then returning to 50/50 acetonitrile/methanol in 10 minutes. Samples were diluted with methanol to an appropriate concentration prior to injection of 10 μ l of solution with an auto injection system operated with oil-free compressed air.

7. Sample Preparation for the Analysis of the Structure of Vitamin D₂ Oxidation Product

The main peaks of oxidation product and vitamin D₂ were collected in multiple 200 μ l runs of the 3000 ppm vitamin D₂ sample which contained 15 ppm riboflavin and was stored in the light for 8 hours. The samples were collected in brown glass vials and evaporated to dryness under a stream of nitrogen. The samples were stored in the dark at 4°C until analyzed.

8. Analysis of Vitamin D₂ Oxidation Product by Mass Spectroscopy (MS), Fourier Transform Infrared Spectroscopy (FTIR) and Ultraviolet Spectroscopy (UV)

Mass spectroscopy by electron impact ionization (EI) was used to analyze vitamin D₂ as obtained from Sigma Chemical Company, the sample of the peak representing vitamin D₂ and the sample of the peak representing the main oxidation product of vitamin D₂.

The sample of the vitamin D₂ oxidation product and vitamin D₂ as obtained from Sigma Chemical Company was dissolved in acetone and dried on a KBr pellet for transmission analysis using a Matteson Cygnus 100 FTIR (Madison, WI). The resolution was 2 cm⁻¹ and 128 scans were performed for each peak.

Samples of vitamin D₂ as obtained from Sigma, the vitamin D₂ peak collected by HPLC and the oxidation product collected by HPLC were dissolved in acetonitrile. The samples were scanned in the UV range from 190 to 268 nm on a Spectronic 1001 spectrophotometer (Milton Roy, Rochester, NY).

RESULTS AND DISCUSSION

1. Riboflavin-Photosensitized Singlet Oxygen Oxidation of Vitamin D₂

Headspace oxygen loss was calculated by subtracting the electronic counts for the oxygen peak of each sample containing vitamin D₂ from the electronic counts of the oxygen peak of the blank containing neither riboflavin nor vitamin D₂. The results for the blank and samples of riboflavin without vitamin D₂ which were stored in the light are shown in Table 2. There was no loss of headspace oxygen over time in the blank. This was expected due to the serum bottles being sealed air-tight.

There was no significant ($p < 0.05$) change with time in the headspace oxygen of the samples containing only riboflavin. This was due to the lack of a substrate for oxidation. Riboflavin can act as a sensitizer to produce singlet oxygen, but the singlet oxygen must have a compound to react with in order for the headspace oxygen to be lost. Oxidation also did not occur in the samples containing vitamin D₂ without riboflavin present (Table 3).

Table 2. Headspace oxygen of samples with riboflavin only stored in the light

riboflavin (ppm)	<u>% headspace oxygen remaining</u>				time (hrs.)
	1	2	4	8	
0	21.42	21.60	21.43	21.74	
5	21.07	21.03	20.96	20.89	
10	21.05	21.03	20.82	20.87	
15	20.98	21.00	20.96	20.87	

Table 3. Headspace oxygen of vitamin D₂ samples without riboflavin stored in the light

vitamin D (ppm)	<u>% headspace oxygen remaining</u>				time (hrs.)
	1	2	4	8	
0	21.42	21.60	21.43	21.74	
3000	21.66	21.26	21.57	21.57	
9000	21.21	21.68	20.95	20.79	

There was no significant ($p < 0.05$) change with time in the headspace oxygen content. Singlet oxygen cannot be produced in this system without sensitizer present and triplet oxygen, a radical compound, cannot react with vitamin D₂ unless vitamin D₂ is transformed into a radical.

Table 4 shows the results for samples containing riboflavin and vitamin D₂ which were stored in the dark. When no light was present, the riboflavin could not be excited to a reactive state so no singlet oxygen could be produced. The headspace oxygen content did not change significantly ($p < 0.05$) in these bottles over time.

Table 4. Headspace oxygen of vitamin D₂ samples with 15 ppm riboflavin stored in the dark

vitamin D (ppm)	<u>% headspace oxygen remaining</u>				time (hrs.)
	1	2	4	8	
0	21.42	21.60	21.43	21.74	
3000	21.40	21.54	21.60	21.55	
9000	21.32	21.19	21.19	20.61	

There was a loss in headspace oxygen when both riboflavin and vitamin D₂ were present in samples stored in the light (Table 5-7). The amount of loss varied depending on the concentrations of vitamin D₂ and riboflavin. There was a significant ($p<0.05$) increase in headspace oxygen loss with an increase in riboflavin or vitamin D₂ concentration and with an increase in time of storage in the light. The smallest loss after one hour of storage in the light, was for the sample containing 3000 ppm vitamin D₂ and 5 ppm riboflavin, 3.71%. The largest loss, 57.3%, was for the sample containing 9000 ppm vitamin D₂ and 15 ppm riboflavin which was stored for 8 hours in the light.

Table 5. Effect of vitamin D₂ concentration on % headspace oxygen loss with 5 ppm riboflavin

vitamin D (ppm)	<u>% headspace oxygen loss *</u>				time (hrs.)
	1	2	4	8	
3000	3.71	5.55	7.15	9.56	
6000	6.91	10.0	15.5	26.2	
9000	8.95	13.8	22.0	39.6	

* significant change with time at $p<0.05$

Table 6. Effect of vitamin D concentration on % headspace oxygen loss with 10 ppm riboflavin

vitamin D (ppm)	<u>% headspace oxygen loss *</u>				time (hrs.)
	1	2	4	8	
3000	6.14	9.15	13.0	17.1	
6000	9.77	15.6	22.4	35.1	
9000	12.6	23.2	32.6	53.2	

* significant change with time at $p < 0.05$

Table 7. Effect of vitamin D concentration on % headspace oxygen loss with 15 ppm riboflavin

vitamin D (ppm)	<u>% headspace oxygen loss *</u>				time (hrs.)
	1	2	4	8	
3000	9.37	14.2	18.6	26.5	
6000	13.1	19.8	29.5	45.2	
9000	16.4	25.1	38.8	57.3	

* significant change with time at $p < 0.05$

In summary, there was no significant loss in headspace oxygen with time when either riboflavin or vitamin D₂ were present alone in the light. Also, there was no significant loss with time in the headspace oxygen of samples containing both vitamin D₂ and riboflavin that were stored in the dark.

However, there was a significant loss in headspace oxygen with time when both vitamin D₂ and riboflavin were present in the samples stored in the light. In order for singlet oxygen to be produced, there must be sensitizer present and light for the sensitizer to be transformed into an excited state. There must be oxygen for the triplet excited sensitizer to react with to form singlet oxygen, which can then oxidize a substrate. If any of the four main requirements of singlet oxygen oxidation, sensitizer, light, oxygen and substrate, are missing then singlet oxygen oxidation cannot occur.

This research demonstrated that when a 12% water/88% acetone solution of vitamin D₂ contained riboflavin, a known sensitizer of singlet oxygen, and was exposed to light in the presence of oxygen, photosensitized singlet oxygen oxidation of vitamin D₂ did occur.

2. Kinetics and Quenching Mechanisms of α -Tocopherol and Ascorbic Acid on Riboflavin-Photosensitized Singlet Oxygen Oxidation of Vitamin D₂

Figure 45 shows the effect of α -tocopherol on riboflavin-photosensitized singlet oxygen oxidation of vitamin D₂. As the concentration of vitamin D₂ increases, more headspace oxygen depletion occurs, but as the concentration of α -tocopherol increases from 0 ppm to 40 ppm and 60 ppm, the depletion of headspace oxygen decreases.

Figure 45. Effect of α -tocopherol on singlet oxygen oxidation of vitamin D₂

The rate of formation of singlet oxygen by 15 ppm riboflavin can be determined from the reciprocal of the intercept of the 0 ppm α -tocopherol regression line of which the units are $\mu\text{mole oxygen/ml headspace in 8 hours}$. The intercept is 7.95×10^{-2} ml headspace/ $\mu\text{mole oxygen}$ which inverted is $12.57 \mu\text{mole oxygen/ml headspace in 8 hours}$. Therefore the rate of formation of singlet oxygen by riboflavin in 12% water/88% acetone is $1.57 \mu\text{mole oxygen/ml headspace/hour}$.

This result is similar to the formation rate of singlet oxygen by 40 ppm FD & C Red No. 3 in ascorbic acid solution which is $1.5 \mu\text{mole oxygen/ml headspace/hour}$ (Yang, 1994) and is 9 times higher than the rate for 4 ppm chlorophyll in methylene chloride in the presence of soybean oil which is $0.17 \mu\text{mole oxygen/ml headspace/hour}$ (Lee, 1987). The rate of formation of singlet oxygen in milk with 0.10 to 0.14 M milkfat was $2.31 \mu\text{mole oxygen/ml headspace/hour}$ (Bradley, 1991), which was approximately 1.5 times higher than for singlet oxygen formation by riboflavin in 12% water/88% acetone.

The intercepts in Figure 45, 7.95×10^{-3} , 3.90×10^{-3} and 4.92×10^{-3} for 0 ppm, 40 ppm and 60 ppm α -tocopherol, respectively, appear to be similar while the slopes vary. This is indicative of singlet oxygen quenching by steady state kinetic analysis. Figure 46 shows the regression line of the ratios of

the slope/Intercepts of the lines in Figure 45 as a function of the concentration of α -tocopherol. The Intercept of the regression line in Figure 46 is equal to the rate of decay of singlet oxygen in the solvent, k_d divided by the rate of reaction of vitamin D₂ with singlet oxygen, k_r . k_d/k_r equals 3.37×10^{-2} . The decay rate, k_d , of singlet oxygen in water is $2.27 \times 10^5 \text{ sec}^{-1}$ and k_d in acetone is 1.96×10^4 so an approximation of the singlet oxygen decay rate in 12% water/88% acetone would be 12% of k_d in water plus 88% of k_d in acetone, 44488 sec^{-1} .

Figure 46. Slope/Intercept ratios of regression lines in Figure 45 as a function of α -tocopherol concentration

Hence, k_r for the reaction of vitamin D₂ with singlet oxygen is $1.32 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$. This is faster than that found for soybean oil in the presence of FD & C Red No. 3 where k_r was $8.5 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$ (Yang,1994) and for the k_r of singlet oxygen with milk containing 0.10 to 0.41 M milkfat, $8.1 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$. The rate constant for the degradation of riboflavin was lower in whole milk, 0.054 hr^{-1} or $1.5 \times 10^{-5} \text{ sec}^{-1}$, than that for skim milk, 0.068 hr^{-1} or $1.89 \times 10^{-5} \text{ sec}^{-1}$ at 2.5°C and in 2690 lux light (Allen and Parks, 1979). The fat in the system can prevent light from penetrating into the milk so that the rate of oxidation is slowed. This is most likely why the rate for the reaction of singlet oxygen with milk is lower than that of vitamin D₂ with singlet oxygen in our system of 12% water/88% acetone.

α -Tocopherol was shown to be an effective quencher of singlet oxygen in a solution of vitamin D₂. The slope of the regression line in Figure 46, 191.0, equals the total quenching rate, k_q , divided by k_r . Therefore, k_q equals $2.52 \times 10^8 \text{ M}^{-1} \text{ sec}^{-1}$ which is 191 times faster than the reaction rate of singlet oxygen with vitamin D₂. This result was similar to literature values. Yang (1994) found a quenching rate of α -tocopherol on singlet oxygen of $4.1 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$, while others have found similar results of up to $6.2 \times 10^8 \text{ M}^{-1} \text{ sec}^{-1}$ for the rate of quenching of singlet oxygen formed by methylene blue in methanol (Foote, 1976).

Figure 47 shows the effects of ascorbic acid on riboflavin-photosensitized singlet oxygen oxidation of vitamin D₂. As was seen with α -tocopherol, headspace oxygen depletion increases with increasing vitamin D₂ and decreases as the concentration of ascorbic acid increases from 0 ppm to 50 ppm and 60 ppm, respectively. The rate of singlet oxygen formation was 1.07 μ mole oxygen/ml headspace/hour in this case. The slopes of the lines are different and the intercepts seem to be similar which is indicative of singlet oxygen quenching by steady state kinetic analysis. Figure 48 shows the regression line of the ratios of slopes/intercepts of Figure 47 as a function of the ascorbic acid concentration.

Figure 47. Effect of ascorbic acid on singlet oxygen oxidation of vitamin D₂

Figure 48. Slope/intercept ratios of the regression lines in Figure 47 as a function of the ascorbic acid concentration

The intercept of the regression line in Figure 48, 2.1×10^{-2} , equals k_d/k_r . k_d is approximately 44488 sec^{-1} for 12% water/88% acetone, so k_r is $2.11 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$. The slope of the same regression line, 10.5, is equal to k_q/k_r , so k_q is $2.23 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$. This means that ascorbic acid is not as effective as α -tocopherol in quenching singlet oxygen during the oxidation of vitamin D₂ in the presence of 15 ppm riboflavin. This may be because ascorbic acid is very heat labile and its stability also depends on the pH of the

solution. The degradation of ascorbic acid increases 1.6-fold with an increase in temperature of 6.5°C (Hsieh and Harris, 1993). The temperature in the light box was 45°C due to the heat produced by the 200 watt bulb, which could have caused the degradation of ascorbic acid. Consequently, less ascorbic acid would be available to act as an antioxidant. The pH of the sample containing 15 ppm riboflavin, 3000 ppm vitamin D₂ and 50 ppm ascorbic acid was 6.40 which also could have affected the stability of the ascorbic acid.

Jung et al. (1995) found that the rate of reaction of ascorbic acid with singlet oxygen, produced by photosensitization with methylene blue, was $5.77 \times 10^8 \text{ M}^{-1} \text{ sec}^{-1}$ in a potassium phosphate buffer at pH 6 and 20°C. The solution was stored for 3 minutes in light at an intensity of 5500 lux. Yang (1994) found a reaction rate for ascorbic acid with singlet oxygen of $3.08 \times 10^8 \text{ M}^{-1} \text{ sec}^{-1}$ in an aqueous solution at pH 7.0 and 25°C. The samples were stored in light at an intensity of 4000 lux for 1 hour and the sensitizer was FD & C Red No. 3 at 40 ppm. The result of Jung et al. (1995) is 26 times and the rate of Yang (1994) is 14 times our quenching rate for ascorbic acid with singlet oxygen. Jung et al. (1995) used a light intensity 1500 lux higher than ours and this could have resulted in greater oxidation of ascorbic acid. An increase in light intensity from 1614 lux to 4842 lux caused an increase in the rate constant for the degradation of ascorbic acid from 0.034 hr^{-1} or $9.44 \times 10^{-6} \text{ sec}^{-1}$ to 0.077 hr^{-1} or $2.14 \times 10^{-5} \text{ sec}^{-1}$ (Gaylord et al.,

1986). Also, an increase of pH from 4.25 to pH 5.0, resulted in a 2-fold increase in the degradation rate of ascorbic acid from $7.51 \times 10^4 \text{ M}^{-1} \text{ hr}^{-1}$ or $20.9 \text{ M}^{-1} \text{ sec}^{-1}$ to $1.18 \times 10^5 \text{ M}^{-1} \text{ hr}^{-1}$ or $32.8 \text{ M}^{-1} \text{ sec}^{-1}$ (Hsieh and Harris, 1993). This could be why the result of Yang (1994) for the rate of reaction of ascorbic acid with singlet oxygen is higher than our ascorbic acid quenching rate. Finally, different sensitizers can give different reaction rates for singlet oxygen and our solution contains vitamin D₂ which competes with the ascorbic acid for reaction with singlet oxygen.

3. Analysis of Vitamin D₂ by HPLC

A sample containing 3000 ppm vitamin D₂ and 15 ppm riboflavin which had been stored in the dark for 8 hours was analyzed by HPLC (Figure 49). The sample was diluted to 10.4 ppm with methanol and the attenuation on the Integrator was 4. There was one peak at a retention time of 9.44 minutes. The other peaks 3.06 and 4.12 minutes are the elution of the acetone, water and methanol as confirmed by a blank run of solvent only (Figure 50).

Figure 49. HPLC chromatogram of 3000 ppm vitamin D₂ sample with 15 ppm riboflavin after storage in the dark for 8 hours

Figure 50. HPLC chromatogram of 12% water/ 88% acetone solvent

When the sample containing 15 ppm riboflavin and 3000 ppm vitamin D₂ was stored in the light for 8 hours and analyzed by HPLC, there were two peaks in the chromatogram at 8.55 and 9.44 minutes (Figure 51).

Figure 51. HPLC chromatogram of 3000 ppm vitamin D₂ sample with 15 ppm riboflavin after storage in the light for 8 hours

The new peak at 8.55 minutes was thought to be a riboflavin photosensitized singlet oxygen oxidation product of vitamin D₂, since it only appeared in samples stored in the light.

Samples with vitamin D₂ only stored in the dark or in the light for 24 hours and diluted to 10.4 ppm vitamin D₂ were analyzed by HPLC at an integrator attenuation of 1 (Figure 52 and 53). Only one peak was observed other than the solvent peak at

10.06 minutes in both samples. A sample containing both 15 ppm riboflavin and 3000 ppm vitamin D₂ which was stored in the dark for 24 hours also showed only one peak at 10.05 minutes, but the same sample which had been stored in the light for 24 hours showed two peaks at 9.10 and 10.06 minutes (Figure 54 and 55). This set of samples meets the requirements of singlet oxygen oxidation cited previously. When no riboflavin is present or if the sample is stored in the dark with riboflavin present, then there is no formation of vitamin D₂ oxidation product. Therefore, singlet oxygen oxidation did not occur.

Samples of 3000 ppm vitamin D₂ alone or 3000 ppm vitamin with 15 ppm riboflavin stored in the light for 8 hours were diluted to 1000 ppm vitamin D₂ with methanol. The samples were analyzed by HPLC at an attenuation of 4 to see if there were any other possible oxidation products that would be detectable at a higher concentration. Figure 56 shows a chromatogram of the sample containing only vitamin D₂. The main peaks besides the solvent peak at 2.91 minutes were those at 7.72 (B), 9.71 (D), 10.88 (E) and 12.84 (F) minutes. In the chromatogram of the sample containing both vitamin D₂ and riboflavin (Figure 57), there were new peaks at 6.27 (A) and 8.97 (C) minutes and all of the previous peaks were lower in area than those in the sample with vitamin D₂ alone (Table 8).

Figure 52. HPLC chromatogram of 3000 ppm vitamin D₂ sample after storage in the dark for 24 hours and dilution to 10 ppm vitamin D₂

Figure 53. HPLC chromatogram of 3000 ppm vitamin D₂ after storage in the light for 24 hours and dilution to 10 ppm vitamin D₂

Figure 54. HPLC chromatogram of 3000 ppm vitamin D₂ with 15 ppm riboflavin after storage in the dark for 24 hours and dilution to 10 ppm vitamin D₂

Figure 55. HPLC chromatogram of 3000 ppm vitamin D₂ with 15 ppm riboflavin after storage in the light for 24 hours and dilution to 10 ppm vitamin D₂

Figure 56. HPLC chromatogram of 3000 ppm vitamin D₂ sample after storage in the light for 8 hours and after dilution to 1000 ppm vitamin D₂

Figure 57. HPLC chromatogram of 3000 ppm vitamin D₂ with 15 ppm riboflavin after storage in the light for 8 hours and dilution to 1000 ppm vitamin D₂

Table 8. Peak areas for HPLC analysis of vitamin D₂ samples

<u>Retention Time (min.)</u>	<u>Area</u>	
	<u>vitamin D₂ alone</u>	<u>with riboflavin</u>
6.27	no peak	70183
7.72	61392	39729
8.97	7006	553612
9.70	155469	91871
10.9	3448376	2309986
12.9	27235	18508

4. Analysis of Main Vitamin D₂ Oxidation Product by MS, UV and FTIR

Several injections of the sample containing vitamin D₂ and riboflavin which had been stored in the light were run on the HPLC and the peaks at 8.97 minutes (oxidation product, peak C) and 10.9 minutes (peak E) were collected. After evaporating to dryness with nitrogen gas, the samples were analyzed by EI MS along with an untreated sample of vitamin D₂ (Figures 58, 59, 60, 61).

Figure 58. Mass Spectrum of vitamin D₂ oxidation product (peak C)

Figure 59. Mass spectrum of vitamin D₂ oxidation product (peak C duplicate)

Figure 60. Mass spectrum of vitamin D₂ HPLC peak E

Figure 61. Mass spectrum of vitamin D₂ control

Both spectra of HPLC peak E at a retention time of 10.9 minutes and untreated vitamin D₂ showed similar fragmentation patterns (Figures 60 and 61). The molecular ion, M⁺, was at m/e = 396, which is the actual molecular weight of vitamin D₂.

The fragment at $m/e = 271$ represents the loss of the side chain of molecular weight = 125, while $m/e = 253$ includes the side chain plus the loss of water. The fragment at $m/e = 136$ represents the molecular weight of the A ring up to position 7 with a molecular formula of $C_9H_{12}O$ and the fragment at $m/e = 118$ includes the loss of water and $C_9H_{12}O$. These are all typical fragments for MS analysis of vitamin D_2 .

The mass spectra for the duplicate oxidation products of vitamin D_2 from HPLC peak C at a retention time of 8.9 minutes are shown in figures 58 and 59. This spectrum also shows fragments at $m/e = 396, 271, 253, 136$ and 118 . However, all of these fragments, except for the oxidized product duplicate at $m/e = 118$ and 136 , have a lower intensity for the oxidized product than the vitamin D_2 sample or HPLC peak E at a retention time of 10.8 minutes (Table 9).

Table 9. Peak intensities as a percentage of the base peak for vitamin D₂, peak E and duplicate samples of peak C representing the oxidation product.

<u>m/e</u>	<u>vitamin D</u>	<u>Relative Intensity</u>		
		<u>peak E</u>	<u>peak C</u>	<u>peak C dup.</u>
118	68.01	100	38.62	100
136	72.23	93.76	33.93	97.14
253	22.86	33.48	6.56	14.74
271	14.40	21.82	5.83	9.15
303	no peak	no peak	4.30	2.06
396	36.51	52.43	15.80	29.48
412	no peak	no peak	2.83	2.49

The M⁺ ion at m/e = 412 is exactly an increase in the molecular weight of oxygen, 16. Only HPLC peak C at 8.9 minutes showed this result, so it most likely is an oxidized product. There are several possibilities for the type of oxidized product which was formed. The product could be a form of hydroxy vitamin D₂, a 5,6 diol, a 7,8 diol or an 5,6 or 7,8 or 10,19 or 22,23 epoxide. These diols and epoxides are at double bonds where oxygen could attack.

A 25-hydroxyergocalciferol has fragments at m/e = 354 and m/e = 59, representing the loss of C₃H₇O, the hydroxyl

group and the 2 end methyl groups on the side chain plus the carbon linking them together (Figure 62, Elliott and Waller, 1972).

Figure 62. 25-Hydroxyergocalciferol

The oxidation peak does not show a fragment at $m/e = 354$. There is a fragment at $m/e = 59$ of intensity 2.25 for HPLC peak C. However, this fragment was also seen in the vitamin D₂ sample at an intensity of 3.58, so there may be a slight contamination of the oxidized product. Also, when there is the presence of a hydroxyl group at position 25, the UV absorbance maximum does not change, since there is no change in the

triene structure (Suda et al., 1969). The HPLC peak C duplicate sample shows a decrease in the absorbance maximum from 264 nm to 250 nm, which indicates that the triene structure has been altered (Figure 63). This leaves the possibility of an epoxide or diol in the triene structure.

Figure 63. UV absorbance of vitamin D₂, peak E and peak C duplicate

The fragment at $m/e = 125$ increases slightly in HPLC peak C, but is about the same in the HPLC peak C duplicate sample as

in the samples of vitamin D₂ and HPLC peak E. This fragment is associated with the loss of the side chain, which indicates that the side chain most likely is not changed in any way. Therefore, the oxidized product is not an epoxide or diol at position 22,23.

A main fragment in the mass spectrum of a 7,8 diol is at $m/e = 277$ which is the molecular weight of the part of the structure from carbon 8 through the end of the side chain (Figure 64, Elliott and Waller, 1972). This peak was not observed in the mass spectra of the oxidized product, so this product is not a 7,8 diol.

Figure 64. Fragmentation Scheme for 7,8-diol of vitamin D₂

The fragment at $m/e = 303$ was present in vitamin D₂-5,6 diol (Figure 65, Elliot and Waller, 1972). It was observed in the oxidized compound, peak C, but not in the samples of vitamin D₂ or HPLC peak E. A 5,6 diol also has a molecular ion peak at $m/e = 430$ for the increase of 2 oxygen atoms with hydrogens.

Figure 65. Fragmentation Scheme of 5,6-diol of vitamin D

The oxidation product in this present study did not show this molecular ion. It is possible that the molecular ion of $m/e = 430$ was not detected by EI MS. There is also a fragment at $m/e = 128$ in 5,6 diols which represents the A ring with two hydroxyl groups, one of which came from the diol hydroxy

group at position 5 (Figure 65). The oxidation product did not show intensities higher than those of vitamin D₂ or HPLC peak E, so there may have not been a diol at position 5,6.

It may be possible that the oxidized product is an epoxide which during EI MS, splits to form a fragment of molecular weight 303, containing the oxygen plus a hydrogen, including the structure from carbon 6 through the end of the side chain (Figure 66). If this split occurred then there would be a fragment of the A ring of molecular weight 112.

Figure 66. Fragmentation scheme for 5,6 epoxide of vitamin D₂

All of the samples show a fragment at $m/e = 112$, except HPLC peak E, with vitamin D₂ and HPLC peak C duplicate having intensities of 0.71 and 0.60, but HPLC peak C had a higher intensity at 1.25. Therefore the oxidized product could be a 5,6 epoxide.

The results of FTIR analysis of oxidized sample (HPLC peak C) and untreated vitamin D₂ showed that there was a difference between the two samples (Figure 67 and 68). There were peaks in the FTIR spectrum of the vitamin D₂ sample at about 834 cm⁻¹, 863 cm⁻¹ and 888 cm⁻¹ and between 600 cm⁻¹ and 800 cm⁻¹ that were not observed in the FTIR spectrum of HPLC peak C. Also the peak at about 1050 cm⁻¹ was much broader in the oxidized vitamin D₂ (HPLC peak C) sample than in the untreated vitamin D₂ sample. The FTIR spectrum of oxidized vitamin D₂ sample did show peaks at 1260 cm⁻¹ and 803 cm⁻¹ which are indicative of the presence of epoxide (Figure 69). Although the FTIR spectrum of the vitamin D₂ sample also shows peaks in these regions (Figure 70), HPLC analysis showed that the vitamin D₂ sample was not pure. Therefore, it is possible that these peaks in the vitamin D₂ sample were due to contamination.

Figure 67. FTIR spectrum of untreated vitamin D₂

Figure 68. FTIR spectrum of oxidized vitamin D₂ (HPLC peak C)

Figure 69. Partial FTIR spectrum of oxidized vitamin D₂ (HPLC peak C)

Figure 70. Partial FTIR spectrum of untreated vitamin D₂

5. Mechanism of Formation of 5,6 epoxide by singlet oxygen oxidation of vitamin D₂.

Figure 71 and 72 show the proposed mechanism for the formation of the 5,6 epoxide from singlet oxygen oxidation of vitamin D₂. Several authors have studied the mechanism behind

Figure 71. Proposed mechanism for the formation of the 5,6 epoxide by singlet oxygen oxidation of vitamin D₂, part 1

Figure 72. Proposed mechanism for the formation of the 5,6 epoxide by singlet oxygen oxidation of vitamin D₂, part 2

the formation of epoxides in photosensitized singlet oxygen oxidation of olefins by the 1,2 cycloaddition reaction (Dewar et al., 1975, Bartlett and Ho, 1974, Schaap and Faler, 1973 and Barlett, 1976). Dewar et al. (1975) found by calculation that the activation energy, for the formation of epoxide from peroxirane intermediate by further reaction with singlet oxygen to form ozone, was about the same as the calculated activation energy for the formation of dioxetane from peroxirane. The calculated activation energy for the formation of epoxide from peroxirane by further reaction with the olefin was about 4 to 5 times smaller than the other two reactions. Sterically nonhindered olefins should then easily follow the latter reaction mechanism and produce mainly epoxide (Dewar et al., 1975).

Peroxiranes of sterically hindered olefins are unable to react with the olefins and must form epoxides by further reaction with singlet oxygen (Dewar et al., 1975), but can also form dioxetanes (Schaap and Faler, 1973, Barlett and Ho, 1974). The formation of epoxide over dioxetane is favored by lower concentrations of olefin and is also dependent on the type of solvent. Barlett (1976) stated that ratio of the formation of epoxide to dioxetane was proportional to the concentration of singlet oxygen, the light intensity and the concentration of sensitizer in dilute solutions. This ratio of epoxide formation to dioxetane formation was inversely proportional to the olefin concentration and the quenching power of the solvent on singlet oxygen.

The mass spectroscopy results in the present study show the increase of only one molecule of oxygen which indicates that the oxidation product of vitamin D₂ was not a dioxetane, but an epoxide. Also there is steric hindrance in the structure of vitamin D₂ due to the ring structure, methylene group and trans nature of the double bonds. Therefore the reaction for the formation of the epoxide most likely followed that shown in Figure 68, by formation of an intermediate peroxirane which reacts with singlet oxygen to form the epoxide and ozone.

CONCLUSIONS

From the results of this research, the following conclusions can be drawn:

1. Singlet oxygen oxidation of vitamin D₂ does occur in the presence of riboflavin in a model system.
2. The rate of singlet oxygen formation in a 12% water/88% acetone solution of vitamin D₂ and 15 ppm riboflavin averaged 1.32 umole oxygen/ml headspace/hr at 4°C.
3. The rate of reaction of singlet oxygen with vitamin D₂ averaged $1.72 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$ at 45°C.
4. α -Tocopherol was an effective antioxidant in retarding riboflavin-photosensitized singlet oxygen oxidation of vitamin D₂ and quenched singlet oxygen at a rate of $2.52 \times 10^8 \text{ M}^{-1} \text{ sec}^{-1}$.
5. Ascorbic acid was not as effective as α -tocopherol in retarding riboflavin-photosensitized singlet oxygen oxidation of vitamin D₂.

Ascorbic acid quenched singlet oxygen at a rate of $2.23 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$.

6. HPLC analysis of the samples, containing both vitamin D₂ and riboflavin, which were stored in the light showed a new peak at 8.97 minutes. Samples stored in the dark or with vitamin D₂ alone in the dark or light did not show this new peak.

7. Mass spectroscopic analysis of the new HPLC peak showed an increase in the molecular weight of 16 which is the exact weight of an atom of oxygen. This indicated that the new HPLC peak was an oxidized product of vitamin D₂.

8. Ultraviolet spectroscopic analysis of the oxidized product showed a decrease in the absorbance maximum compared to untreated vitamin D₂ which indicates an interaction with the triene structure of vitamin D₂.

9. Fourier transform infrared spectroscopic analysis showed that the oxidized product was different from untreated vitamin D₂ in the fingerprint region. FTIR also showed peaks representing those of an epoxide at 1260 cm^{-1} and 803 cm^{-1} .

10. Mass spectroscopic analysis gave strong evidence that the oxidized sample was a 5,6 epoxide of vitamin D₂.

LIST OF REFERENCES

- Allen, C. and Parks, O.W. Photodegradation of Riboflavin in Milks Exposed to Fluorescent Light. *Journal of Dairy Science*. 62:1377. 1979.
- Bradley, D.G. Riboflavin Photosensitized Singlet Oxygen Oxidation in Milk Products. Ph.D. Dissertation. The Ohio State University. Columbus, Ohio. 1991.
- Chin, H.W. and Lindsay, R.C. Ascorbate and Transition Metal Mediation of Methanethiol Oxidation to Dimethyl Disulfide and Dimethyl Trisulfide. *Food Chemistry*. 49:387. 1994.
- Choe, E. and Min, D.B. Interaction Effects of Chlorophyll, β -Carotene and Tocopherol on the Photooxidative Stabilities of Soybean Oil. *Foods and Biotechnology*. 1:104. 1992.
- Cooperman, J.M. and Lopez, R. Riboflavin In Handbook of Vitamins Nutritional Biochemical and Clinical Aspects. ed. Machlin, L.J. Marcel Dekker, Inc. New York. pp. 299-327. 1984.
- Dimick, P.S. Photochemical Effects on Flavor and Nutrients of Fluid Milk. *Canadian Institute of Food Science and Technology Journal*. 15:247. 1982.
- Du, Z. and Bramlage, W. Malondialdehyde Oxidation by Hydrogen Peroxide and by Light Excited Riboflavin in Model Systems. *Journal of Food Science*. 58:925. 1993.
- Elliott, W.H. and Waller, G.R. Vitamins and Cofactors In Biochemical Applications of Mass Spectrometry. ed. Waller, G.H. Wiley Interscience. New York. Chp. 18. 1972.

Foote, C.S. Photosensitized oxidation and Singlet Oxygen: Consequences in Biological Systems in Free Radicals in Biology. Pyro, W.A. (Ed.) Academic Press, NY. Vol. 2 p. 85. 1976.

Furuya, E.M. and Warthesen, J.J. Influence of Initial Riboflavin Content on Retention in Pasta During Photodegradation and Cooking. *Journal of Food Science*. 49:984. 1984.

Furuya, E.M., Warthesen, J.J. and Labuza, T.P. Effects of Water Activity, Light Intensity and Physical Structure of Food on the Kinetics of Riboflavin Photodegradation. *Journal of Food Science*. 49:525. 1984.

Gaylord, A.M., Warthesen, J.J. and Smith, D.E. Influence of Milk Fat, Milk Solids and Light Intensity on the Light Stability of Vitamin A and Riboflavin in Lowfat Milk. *Journal of Dairy Science*. 69:2779. 1986.

Gollnick, K. and Kuhn, H.J. Ene Reactions with Singlet Oxygen in Singlet Oxygen. ed. Wasserman, H.H. and Murray, R.W. Academic Press. New York. pp. 287-427. 1979.

Gordon, D.T., Roberts, G.L. and Heintz. Thiamin, Riboflavin and Niacin Content and Stability in Pacific Coast Seafoods. *Journal of Agricultural and Food Chemistry*. 27:483. 1979.

Head, M.K. and Hansen, A.P. Stability of L-Ascorbic Acid Added to Whole, Chocolate and Low Fat Milk. *Journal of Dairy Science*. 62:352. 1979.

Hsieh, Y.H. and Harris, N.D. Effect of Sucrose on Oxygen Uptake of Ascorbic Acid In a Closed Aqueous System. *Journal of Agricultural and Food Chemistry*. 41:259. 1993.

Huang, A.S. and Byerly, D.S. Sorption Isotherms and Light Stabilities of Aluminum Laked Riboflavin 5'-Phosphate. *Journal of Food Science*. 57:245. 1992.

Jacobus, C.H., Holick, M.F., Shao, Q., Chen, T.C., Holm, I.A., Kolodny, J.M., Fuleihan, G.E.H. and Seely, E.W. New England Journal of Medicine. 326:1173. 1992.

Jaffe, G.M. Vitamin C in Handbook of Vitamins Nutritional Biochemical and Clinical Aspects. ed. Machlin, L.J. Marcel Dekker, Inc. New York. pp. 199-244. 1984.

Johnson, J.R., Braddock, R.J. and Chen, C.S. Kinetics of Ascorbic Acid Loss and Nonenzymatic Browning in Orange Juice Serum: Experimental Rate Constants. Journal of Food Science. 60:502. 1995.

Jung, M.Y., Kim, S.K. and Kim, S.Y. Riboflavin sensitized photooxidation of ascorbic acid: kinetics and amino acid effects. Food Chemistry. 53:397. 1995.

Jung, M.Y., Lee, H.O., Yoon, S.H. and Min, D.B. Effects of Ascorbic Acid on the Off-Flavor Formation in Skim Milk Exposed to Light. unpublished. 1994.

Kanner, J.D. and Fennema, O. Photooxidation of Tryptophan in the Presence of Riboflavin. Journal of Agricultural and Food Chemistry. 35:71. 1987.

Kearsley, M.W. and Rodriguez, N. The Stability and Use of Natural Colours in Foods: Anthocyanin, β -Carotene and Riboflavin. Journal of Food Technology. 16:421. 1981.

Kennedy, J.F., Rivera, Z.S., Lloyd, L.L., Warner, F.P. and Jumel, K. L-Ascorbic Acid Stability in Aseptically Processed Orange Juice in TetraBrik Cartons and the Effect of Oxygen. Food Chemistry. 45:327. 1992.

Khattak, A.B. and Klopfenstein, C.F. Effects of Gamma Irradiation on the Nutritional Quality of Grain and Legumes. I. Stability of Niacin, Thiamin and Riboflavin. Cereal Chemistry. 66:169. 1989.

Kirk, J., Dennison, D., Kokoczka, P. and Heldman, D. Degradation of Ascorbic Acid in a Dehydrated Food System. *Journal of Food Science*. 42:1274. 1977.

Korycka-Dahl, M. and Richardson, T. Activated Oxygen Species and Oxidation of Food Constituents. *Critical Reviews in Food Science and Nutrition*. 10:209. 1978.

Kreutler, P. A. and Czajka-Narins, D. M. Nutrition in Perspective. 2nd edition. Prentice Hall. New Jersey. p. 190. 1987.

Labuza, T.P. Kinetics of Lipid Oxidation in Foods. *Critical Reviews in Food Technology*. 10:355. 1971.

Lee, E.C. Effects of Chlorophyll, Carotene and Tocopherol on the Singlet Oxygen Oxidation of Soybean Oil. Ph.D. Dissertation. The Ohio State University. 1987.

Machlin, L.J. Vitamin E in Handbook of Vitamins Nutritional Biochemical and Clinical Aspects. ed. Machlin, L.J. Marcel Dekker, Inc. New York. pp. 99-145. 1984.

Maeda, E.E. and Mussa, D.M.D.N. The Stability of Vitamin C (L-Ascorbic Acid) in Bottled and Canned Orange Juice. *Food Chemistry*. 22:51. 1986.

Miller, B.E. and Norman, A.W. Vitamin D in Handbook of Vitamins Nutritional Biochemical and Clinical Aspects. ed. Machlin, L.J. Marcel Dekker, Inc. New York. p.80. 1984.

Moriuchi, S., Tsuruki, F., Otawara, Y., Hosoya, N., Yamada, S., Nakayama, K. and Takayama, H. Biological Activity of the Endoperoxides Derived from Vitamin D Derivatives by Dye-Sensitized Photooxidation. *Journal of Nutritional Science and Vitaminology*. 25:455. 1979.

Nalm, M., Wainish, S., Zehavi, U., Pelag, H., Rouseff, R.L. and Nagy, S. *Journal of Agricultural and Food Chemistry*. 41:1355. 1993.

Palanuk, S.L. and Warthesen, J.J. The Kinetics of Lumichrome in Skim Milk Using Nonlinear Regression Analysis. Food Chemistry. 27:115. 1988.

Palanuk, S.L., Warthesen, J.J. and Smith, D.E. Effect of Agitation, Sampling Location and Protective Films on Light Induced Riboflavin Loss in Skim Milk. Journal of Food Science. 53:436. 1988.

Parish, D.B. Determination of Vitamin D in Foods: A Review CRC Critical Reviews in Food Science and Nutrition. 12:29. 1979.

Parks, O.W. and Allen, C. Photodegradation of Riboflavin to Lumichrome in Milk Exposed to Sunlight. Journal of Dairy Science. 60:1038. 1977.

Rawls, H.R. and Van Santen, P.J. A possible Role for Singlet Oxygen in the Initiation of Fatty Acid Autooxidation. Journal of the American Oil Chemists Society. 47:121. 1970.

Reddy, G.S. and Tserng, K.Y. 24,25,28-Trihydroxyvitamin D₂ and 24,25,26-Trihydroxyvitamin D₂: Novel Metabolites of Vitamin D₂. Biochemistry. 29:943. 1990.

Renken, S.A. and Warthesen, J.J. Vitamin D Stability in Milk. Journal of Food Science. 58:5522. 1993.

Rosenthal, I. Photooxidation of Foods in Singlet Oxygen Polymers and Biomolecules. ed. Friemer, A.A. CRC Press. Boca Raton, Florida. Vol. 4. pp. 145-163. 1985.

Saguy, I. Kopelman, I.J. and Mizrahi, S. Simulation of Ascorbic Acid Stability During Heat Processing and Concentration of Grapefruit Juice. Journal of Food Process Engineering. 2:213. 1978.

Sahbaz, F. and Somer, G. Photosensitized Decomposition of Ascorbic Acid in the Presence of Riboflavin. Food Chemistry. 46:177. 1993.

Sahbaz, F. and Somer, G. The Effect of Citrate Anions on the Kinetics of Cupric Ion Catalyzed Oxidation of Ascorbic Acid. Food Chemistry. 47:345. 1993.

Salto, I. and Matsuura, T. The Oxidations of Electron Rich Aromatic Compounds in Singlet Oxygen. ed. Wasserman, H.H. and Murray, R.W. Academic Press. New York. pp. 511-574. 1979.

Sattar, A. and deMan, J.M. Photooxidation of Milk and Milk Products: A Review. CRC Critical Reviews in Food Science and Nutrition. 7:13 1975.

Schaap, A.P. and Zaklika, K.A. 1,2-Cycloaddition Reactions of Singlet Oxygen in Singlet Oxygen. ed. Wasserman, H.H. and Murray, R.W. Academic Press. New York. pp. 173-242. 1979.

Spirichev, V.B. and Sergeev, I.N. Vitamin D: Experimental Research and Its Practical Application. World Reviews of Nutrition and Dietetics. 56:173. 1988.

Suda, T., Deluca, H.F., Schnoes, H.K., and Blunt, J.W. The Isolation and Identification of 25-Hydroxyergocalciferol. Biochemistry. 8:3515. 1969.

Takeuchi, A., Okano, T., Tsugawa, N., Tasaka, Y., Kobayashi, T., Kodama, S. and Matsuo, T. Effects of Ergocalciferol Supplementation on the Concentration of Vitamin D and Its Metabolites in Human Milk. Vitamins. 8:1639. 1989.

Vianni, R. Photooxidation Studies on Soybean Oil. Ph.D. Thesis. Michigan State University. East Lansing, Michigan. 1980.

Wasserman, H.H. and Lipshutz, B.H. Reactions of Singlet Oxygen with Heterocyclic Systems in Singlet Oxygen. ed. Wasserman, H.H. and Murray, R.W. Academic Press. New York. pp. 429-509. 1979.

Yang, W.T.S. Effects of Synthetic Food Colorants on Singlet Oxygen Oxidation of Foods. Ph.D. Dissertation. The Ohio State University. Columbus, Ohio. 1994.