Riboflavin Photosensitized Singlet Oxygen Oxidation of Vitamin D

JOAN M. KING and DAVID B. MIN

ABSTRACT

Samples containing various levels of vitamin D and riboflavin, with and without ascorbic acid or a-tocopherol were prepared in a model system. Samples were stored in the light or in the dark at 45°C for up to 8 h. Headspace oxygen was determined by gas chromatography with thermal conductivity detection. Oxidation of vitamin D was not observed in samples without riboflavin stored in the light nor in samples with riboflavin stored in the dark. Vitamin D with riboflavin was oxidized under light. α-Tocopherol quenched singlet oxygen at a rate of 2.52×10^8 M^{-1} sec^{-1}, whereas ascorbic acid quenched singlet oxygen at a rate of 2.23×10^7 M^{-1} sec^{-1}.

KEYWORDS: singlet oxygen, oxidation, vitamin D, riboflavin, photosensitized

INTRODUCTION

PHOTOCHEMICAL DESTABILIZATION OF MILK HAS BEEN STUDIED for several years. Many reviews have been published concerning the effects of photochemical oxidative changes on the flavor and nutritive value of milk (Korycka-Dahl and Richardson, 1980; Bradley, R.L., 1980; Dimick, 1982; Allen and Joseph, 1985). Riboflavin was shown to be a main factor in the development of off-flavor in milk (Aurand et al., 1966). Riboflavin, when exposed to light, transforms to an excited triplet state which can then oxidize methionine in milk to form methional (Dimick, 1982). Methional was a confirmed component of light-induced off-flavor in milk and lumichrome was shown to be the product of the photo-degradation of riboflavin (Allen and Parks, 1975, 1977).

Photo-degradation of lipids also contributed to the off-flavor as well as to the loss of essential fatty acids in milk. Singlet oxygen was proposed as an initiator of fatty acid oxidation and was found to react 1400 times faster than triplet oxygen with linoleic acid (Rawls and van Santen, 1970). Singlet oxygen was detected in milk by electron spin resonance spectroscopy and had formed in the presence of riboflavin (Berliner et al., 1994).

The Photo-degradation of other B vitamins, as well as vitamin A, vitamin C, carotene and folic acid have been examined (Sattar and de Man, 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 caused loss of vitamin D in skim milk. Vitamin D is unstable when exposed to light and can undergo oxidation in air. However, the contribution of riboflavin to the photosensitized singlet oxygen oxidation of vitamin D has not been proven. The mechanism and kinetics of riboflavin-photosensitized oxidation of vitamin D have not been reported.

Sensitizers are required for the formation of singlet oxygen, and riboflavin, which is in milk, is a known sensitizer. Our first objective was to prove the hypothesis that riboflavin-photosensitized singlet oxygen oxidation of vitamin D occurs. The second objective was to determine the kinetics and mechanisms for quenching singlet oxygen oxidation of vitamin D in order to provide a basis for retarding or preventing such oxidation.

Author King, formerly with Ohio State Univ., is currently associated with the Dept. of Food Science, 111 Food Science Bldg., Louisiana State Univ., Baton Rouge, LA 70803-4200. Author Min is with the Dept. of Food Science & Technology, The Ohio State University, Columbus, OH 43202. Direct inquiries to Dr. Joan M. King.

MATERIALS & METHODS

Materials

Analytical reagent grade acetone (Mallinckrodt Specialty Chemical Co., Paris, KY) was used for sample preparation. Ergocalciferol (vitamin D_2), riboflavin, α-tocopherol and ascorbic acid were obtained from Sigma Chemical Company (St. Louis, MO). High purity helium and oxygen were obtained from the chemical store at The Ohio State University.

Determination of riboflavin-photosensitized singlet oxygen oxidation of vitamin D_2

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. Each riboflavin solution (16 mL) was added to 25-mL serum bottles containing 0, 3000, 6000 or 9000 ppm of vitamin D_2. The bottles were capped airtight 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 D_2 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 (General Electric Company, Cleveland, OH) at an intensity of 4,000 lux as measured with a light meter (Weston Instruments, Newark, New Jersey). The samples were placed on a motor-driven plastic disk (Talboys Engineering Co., Emerson, NJ) rotating at 60 rpm for uniform light exposure (Yang, 1994). The headspace of each sample was analyzed every 1, 2, 4 and 8 h for oxygen content after equilibration to 20°C in a water bath for 10 to 15 min.

Sample preparation for analysis of kinetics and quenching mechanisms of α-tocopherol and ascorbic acid

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. Each riboflavin solution (16 mL) was added to 25-mL serum bottles containing 0, 3000, 6000 or 9000 ppm of vitamin D_2. The bottles were capped airtight 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 D_2 was completely dissolved. The aluminum foil was removed prior to storage. Samples were stored at 45°C in the light under the same conditions as before. The headspace of each sample was analyzed after 8 h for oxygen content after equilibration to 20°C in a water bath for 10 to 15 min.

Headspace oxygen analysis

Gas chromatographic (GC) headspace analysis was used to determine the extent of vitamin D_2 oxidation. A Hewlett Packard 5890 (Avondale, PA) GC with thermal conductivity detector 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. Headspace gas (100 mL) 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) at The Ohio State University. Significance was defined at $p \leq 0.05$.

Conversion of electronic counts to oxygen content

Parker (1987) reported that 100 μL of air contained 0.020946 mL of oxygen at standard temperature and pressure, which represents 8.45×10^{-7} moles O_2 (n). The moles of oxygen in the headspace of each sample were calculated by first subtracting the electronic counts for the sample from the electronic counts of a blank sample containing only 12% water/88% acetone. Then the result was divided by the electronic counts for the daily air reference and multiplied by n. This value was converted to μmoles of oxygen/mL headspace for each replicate by multiplying by 1×10^6 μmoles/mole and dividing by 0.1 mL, the volume of headspace analyzed. Replicate results were averaged for graphing.

RESULTS & DISCUSSION

Riboflavin-photosensitized singlet oxygen oxidation of vitamin D_2

Headspace oxygen loss was calculated by subtracting the electronic counts for the oxygen peak of each sample containing vitamin D_2 from the electronic counts of the oxygen peak of the blank containing neither riboflavin nor vitamin D_2. There was no loss of headspace oxygen over time in the blank of 12% water/88% acetone, which had a headspace oxygen content of 21.6 +/- 0.16%. This was expected due to the serum bottles being sealed airtight.

There was no change ($p \geq 0.05$) with time in the headspace oxygen of the samples containing 5 ppm, 10 ppm or 15 ppm riboflavin, which had been stored in the light (data not shown). 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 samples stored in the light which contained vitamin D_2 without riboflavin present. There was no change ($p \geq 0.05$) with time in the headspace oxygen content (data not shown). Singlet oxygen could not be produced in this system without sensitizer present, and triplet oxygen, a radical compound, could not react with vitamin D_2 unless vitamin D_2 was transformed into a radical.

When no light was present, the riboflavin could not be excited to a reactive state so no singlet oxygen could be produced when both riboflavin and vitamin D were stored in solution in the dark (Table 1). The headspace oxygen content did not change ($p \geq 0.05$) in these bottles over time. There was a loss in headspace oxygen when both riboflavin and vitamin D_2 were present in samples stored in the light (Table 2). The amount of loss varied depending on concentrations of vitamin D_2 and riboflavin. There was an increase ($p \geq 0.05$) in headspace oxygen loss with an increase in riboflavin or vitamin D_2 concentration and with an increase in time of storage in the light. The smallest loss after 1 h of storage in the light, was 3.71% for the sample containing 3000 ppm vitamin D_2 and 5 ppm riboflavin. The largest loss, 57.3%, was for the sample containing 9000 ppm vitamin D_2 and 15 ppm riboflavin which was stored for 8 h in the light.

Thus, there was no loss in headspace oxygen when either riboflavin or vitamin D_2 were present alone in solution in the light. Also, there was no loss in samples containing both vitamin D_2 and riboflavin that were stored in the dark. However, there was significant loss when both vitamin D_2 and riboflavin were present in samples stored in the light. In order for singlet oxygen to be produced, there must be a 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 4 main requirements, sensitizer, light, oxygen and substrate, are missing, then singlet oxygen oxidation cannot occur.

These results demonstrated that when a 12% water/88% acetone solution of vitamin D_2 contained riboflavin, a known sensitizer of singlet oxygen, and was exposed to light in the presence of oxygen, photosensitized singlet oxygen oxidation of vitamin D_2 occurred.

Table 1—Headspace oxygen of vitamin D_2 samples with 15 ppm riboflavin stored in the dark from 1 to 8 hours

Time (h)	Vitamin D2 (ppm)	Headspace oxygen remaining* (%)
1	3000	21.40
2	3000	21.54
4	3000	21.60
8	3000	21.55
1	9000	21.32
2	9000	21.19
4	9000	21.19
8	9000	20.61

* no significant change with time

Table 2—Effect of vitamin D_2 and riboflavin concentrations on % headspace oxygen loss during storage in the light from 1 to 8 hours

Time (h)	Vitamin D2 (ppm)	Riboflavin (ppm)	Headspace oxygen loss* (%)
1	3000	5	3.71
2	3000	5	5.55
4	3000	5	7.15
8	3000	5	9.56
1	6000	5	6.91
2	6000	5	10.0
4	6000	5	15.5
8	6000	5	26.2
1	9000	5	8.95
2	9000	5	13.8
4	9000	5	22.0
8	9000	5	39.6
1	3000	10	6.14
2	3000	10	9.15
4	3000	10	13.0
8	3000	10	17.1
1	6000	10	9.77
2	6000	10	15.6
4	6000	10	22.4
8	6000	10	35.1
1	9000	10	12.6
2	9000	10	23.2
4	9000	10	32.6
8	9000	10	53.2
1	3000	15	9.37
2	3000	15	14.2
4	3000	15	18.6
8	3000	15	26.5
1	6000	15	13.1
2	6000	15	19.8
4	6000	15	29.5
8	6000	15	45.2
1	9000	15	16.4
2	9000	15	25.1
4	9000	15	38.8
8	9000	15	57.3

* significant change with time at $p<0.05$

Kinetics and quenching mechanisms of α-tocopherol and ascorbic acid

As the concentration of vitamin D_2 increased, more headspace oxygen depletion occurred, but as the concentration of α-tocopherol increased from 0 ppm to 40 ppm and 60 ppm, the depletion of headspace oxygen decreased (Fig. 1). 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 μmole oxygen/mL headspace in 8 h (Fig. 1). The intercept was 7.95×10^{-2} mL headspace/μmole oxygen which inverted is 12.57 μmole oxygen/mL headspace in 8 h. Therefore, the rate of formation

of singlet oxygen by riboflavin in 12% water/88% acetone was 1.57 μmole oxygen/mL headspace.hr.

This result was similar to the formation rate of singlet oxygen by 40 ppm FD & C Red No. 3 in ascorbic acid solution which was 1.5 μmole oxygen/mL headspace.hr (Yang, 1994). Also it was 9 times higher then the rate for 4 ppm chlorophyll in methylene chloride in the presence of soybean oil which was 0.17 μmole oxygen/mL headspace.hr (Lee and Min, 1988). The rate of formation of singlet oxygen in milk with 0.10 to 0.14 M milkfat was 2.31 μmole oxygen/mL headspace.hr (Bradley, 1991), which was 1.5 times higher than for singlet oxygen formation by riboflavin in 12% water/88% acetone.

The intercepts (Fig. 1) 7.95×10^{-2}, 8.22×10^{-2} and 7.82×10^{-2} for 0 ppm, 40 ppm and 60 ppm α-tocopherol, respectively, are statistically the same ($p \geq 0.05$) while the slopes vary. This is indicative of singlet oxygen quenching (Foote, 1979). The slopes (S) and intercepts (I) of the regression lines (Fig. 1) were compared (Table 3). A plot of S/I as a function of the concentration of α-tocopherol can be used to determine the reaction rate of vitamin D_2 with singlet oxygen (Foote, 1979). The intercept of this plot is equal to the rate of decay of singlet oxygen in the solvent, k_d divided by the rate of reaction of vitamin D_2 with singlet oxygen, k_r. The regression line was $y=3.37 \times 10^{-2} + 191.0x$ ($R^2=93.9$), so k_d/k_r equals 3.37×10^{-2}. The decay rate, k_d, of singlet oxygen in water is 2.27×10^5 sec^{-1} (Rodgers and Snowden, 1982) and k_d in acetone is 1.96×10^4 sec^{-1} (Byteva and Gurinovitch, 1979) 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}. Hence, k_r for the reaction of vitamin D_2 with singlet oxygen is 1.32×10^6 M^{-1} sec^{-1}.

This rate was similar to that reported for the k_r of singlet oxygen with milk containing 0.10 to 0.41 M milkfat, 8.1×10^5 M^{-1} sec^{-1} (Bradley, 1991) and with methionine, 3×10^7 M^{-1} sec^{-1} (Nilsson et al., 1972). The rate constant for the degradation of riboflavin was lower in whole milk, 0.054 hr^{-1} or 1.5×10^{-5} sec^{-1}, than that for skim milk, 0.068 hr^{-1} or 1.89×10^{-5} 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 (Head and Hansen, 1979). This could be one reason why the rate for the reaction of singlet oxygen with milk is lower than that of vitamin D_2 with singlet oxygen in our system of 12% water/88% acetone. Another reason is that the double bond structure of vitamin D_2 is naturally in the conjugated form, whereas that of the unsaturated fatty acids in milk fat is not. Conjugated bonds are at a lower energy level than non-conjugated bonds and could more easily react.

Table 3—Slopes and intercepts of the regression lines of Fig. 1 for determining quenching rate of α-tocopherol on singlet oxygen oxidation of vitamin D_2

α-Tocopherol ($\times 10^{-4}$ M)	Slope (S) ($\times 10^{-3}$ M-mL headspace/μmole O_2)	Intercept (I) ($\times 10^{-2}$ mL headspace/μmole O_2)	S/I ($\times 10^{-2}$ M)
0.00	2.78	7.95	3.50
1.07	3.90	8.22	4.74
1.60	4.92	7.82	6.29

α-Tocopherol was shown to be an effective quencher of singlet oxygen in a solution of vitamin D_2. The slope of the plot of S/I as a function of the concentration of α-tocopherol, 191.0, equals the total quenching rate, k_q, divided by k_r. Therefore, k_q equals 2.52×10^8 M^{-1} sec^{-1} which is 191 times faster than the reaction rate of singlet oxygen with vitamin D_2. This result was similar to published values. Yang (1994) found a quenching rate of α-tocopherol on singlet oxygen of 4.1×10^7 M^{-1} sec^{-1}, and others have reported similar results of up to 6.2×10^8 M^{-1} sec^{-1} for the rate of quenching of singlet oxygen formed by methylene blue in methanol (Foote, 1976).

As was seen with α-tocopherol, headspace oxygen depletion increased with increasing vitamin D_2 and decreased as the concentration of ascorbic acid increased from 0 ppm to 50 ppm and 60 ppm, respectively (Fig. 2). The rate of singlet oxygen formation was 1.07 μmole oxygen/mL headspace.hr. The slopes of the lines were different and the intercepts were statistically the same ($p \geq 0.05$) which is indicative of singlet oxygen quenching (Foote, 1979). The slopes and intercepts of the regression lines (Fig. 2) were compared (Table 4). The regression line of the plot of S/I as a function of the ascorbic acid concentration was $y=2.10 \times 10^{-2}+10.5x$ (R2=0.856). The intercept of this regression line, 2.1×10^{-2}, equals k_d/k_r. k_d is approximately 44488 sec^{-1} for 12% water/88% acetone, so k_r was 2.11×10^6 M^{-1} sec^{-1}. The slope of the same regression line, 10.5, is equal to k_q/k_r, so k_q was 2.23×10^7 M^{-1} sec^{-1}.

Hence, ascorbic acid was less effective than α-tocopherol in quenching singlet oxygen during the oxidation of vitamin D_2 in the presence of 15 ppm riboflavin. This may be because the stability of ascorbic acid depends on the pH (Hsieh and Harris, 1993). The pH of the sample containing 15 ppm riboflavin, 3000 ppm vitamin D_2 and 50 ppm ascorbic acid was 6.40 which also could have affected the stability of the ascorbic acid. The initial rate of riboflavin-photosensitized decomposition of 1.14×10^{-3} M ascorbic acid at pH 6.0, in the pres-

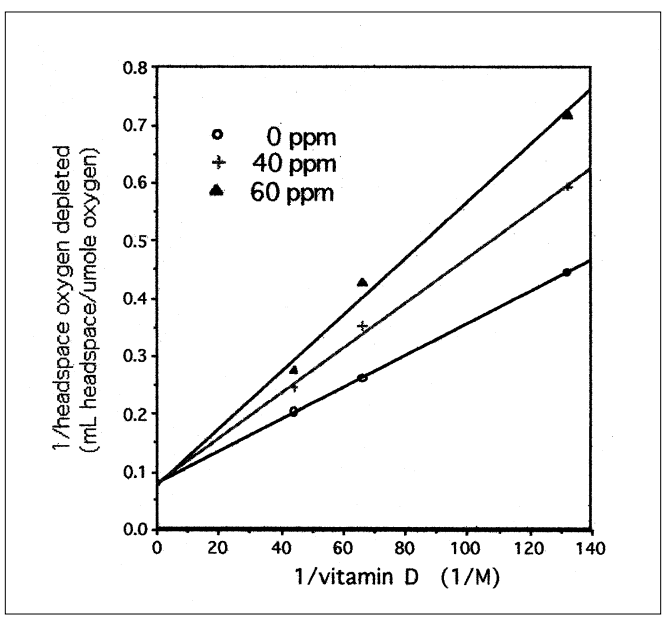

Figure 1—Effect of α-tocopherol on singlet oxygen oxidation of vitamin D_2

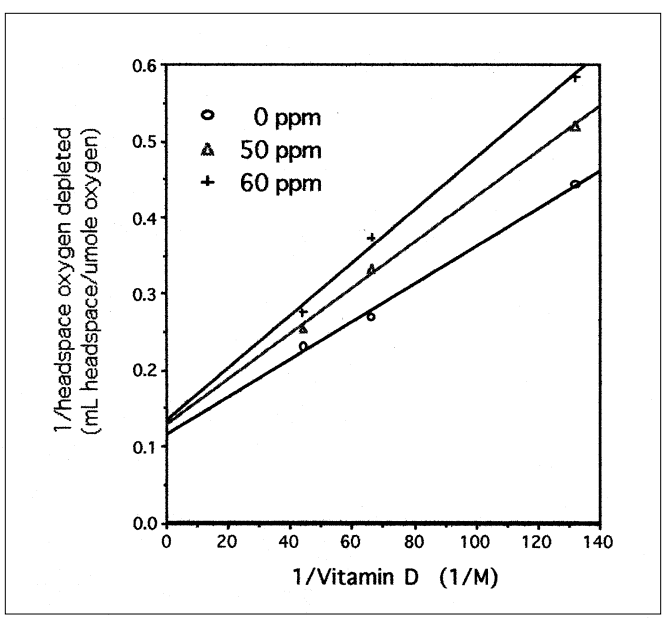

Figure 2—Effect of ascorbic acid on singlet oxygen oxidation of vitamin D_2

Table 4—The slopes and intercepts of the regression lines of Fig. 2 for determining the quenching rate of ascorbic acid on singlet oxygen oxidation of vitamin D_2

Ascorbic acid ($\times 10^{-4}$ M)	Slope (S) ($\times 10^{-3}$ M-mL headspace/imole O_2)	Intercept (I) ($\times 10^{-2}$ mL headspace/imole O_2)	S/I ($\times 10^{-2}$ M)
0.00	2.46	11.6	2.12
1.07	2.98	12.9	2.31
1.60	3.42	13.5	2.53

ence of 9.44 ppm riboflavin, was 9.26 x 10^7 M/min at 25°C. (Sahbaz and Somer, 1993). At pH 2.5 the initial rate of decomposition of ascorbic acid was 10 times less, 9.2 x 10^6 M/min. All solutions had been exposed to a 100-watt tungsten-filamented incandescent lamp.

Jung et al. (1995) found that the rate of reaction of ascorbic acid with singlet oxygen, produced by photosensitization with 6 ppm methylene blue, was 5.77 x 10^8 M^{-1} sec^{-1} in a potassium phosphate buffer at pH 6 and 20°C. The solution was stored for 3 min in light at an intensity of 5500 lux. Yang (1994) reported a reaction rate for ascorbic acid with singlet oxygen of 3.08 x 10^8 M^{-1} 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 h and the sensitizer was FD & C Red No. 3 at 40 ppm. The result of Jung et al. (1995) was 26 times and the rate of Yang (1994) was 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 x 10^{-6} sec^{-1} to 0.077 hr^{-1} or 2.14 x 10^{-5} sec^{-1} (Gaylord et al., 1986). Also, an increase from pH 4.25 to pH 5.0 resulted in a 2-fold increase in the degradation rate of ascorbic acid from 7.51 x 10^4 M^{-1} hr^{-1} or 20.9 M^{-1} sec^{-1} to 1.18 x 10^5 M^{-1} hr^{-1} or 32.8 M^{-1} sec^{-1} (Hsieh and Harris, 1993). This could be the reason our ascorbic acid quenching rate was lower than the result of Yang (1994). Finally, different sensitizers can give different reaction rates for singlet oxygen and our solution contained vitamin D_2 which competes with ascorbic acid for reaction with singlet oxygen.

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