IRRADIATION EFFECTS

Effect of Certain Vitamins and Antioxidants on Irradiation-Induced Autoxidation of Methyl Linoleate

BARBARA HINDERER POLISTER and JAMES F. MEAD
School of Medicine, University of California, Los Angeles, Calif.

In an attempt to ascertain the effect of irradiation upon emulsions containing unsaturated fatty acids in the presence of vitamins and other essential metabolites, several of these substances were irradiated with 1000 roentgens in admixture with methyl linoleate emulsions. Vitamin A, ascorbic acid, glutathione, and cysteine were largely destroyed, depending on their concentration relative to linoleate. Calciferol was not affected, although it showed a low order of antioxidant activity. Tocopherol and a commercial antioxidant (Ionol) were effective antioxidants at concentrations so low that the extent of their destruction was not measured. Catalase had no effect. Knowledge has been gained on destruction of vitamins to be expected during radiation sterilization of fatty foods, and antioxidants preventing this destruction can be suggested.

When linoleic acid is exposed to ionizing radiation, a chain reaction is induced which is similar to autoxidation in all respects except initiation (*11*). This reaction was of particular interest, in that it concerned a compound which is universally present in the animal body and serves as an example of an irradiation-induced chain reaction which might conceivably take place in vivo. It was also important in connection with studies on the destruction of essential metabolites during the irradiation of foodstuffs. In a continuation of this research, an investigation has been made of the effect of vitamins and other naturally occurring antioxidants on the course of the reaction.

In the previous experiments, solution of the linoleic acid was effected at pH 8.5 or 9, but in the present it was thought that an emulsion of methyl linoleate would be more suitable, especially because it would more nearly duplicate conditions existing in tissues. Consequently some time was spent finding the most suitable emulsifying agents and conditions for irradiation of the emulsions.

The effect of catalase was then ascertained, as it was thought that hydrogen peroxide might play some part in the reaction. The vitamins studied were those which, either because of their reducing properties or their presence in normal body lipides, might be expected to have some effect: vitamin A, tocopherol, calciferol, and ascorbic acid. Two sulfhydryl reducing agents, cysteine and glutathione, and a commercial antioxidant, Ionol (2,6-di-*tert*-butyl-4-methylphenol) were also studied.

The irradiation of vitamin A in various media has received some attention. In milk and butter and in aqueous and nonaqueous solution or suspension both carotene and vitamin A are destroyed with an ionic yield approaching unity (*5*, *6*, *9*). In liver or serum, however, these substances are apparently protected (*5*, *14*, *17*).

Ascorbic acid destruction by ionizing radiation has been the subject of some study. Proctor and coworkers (*12*, *13*) have shown that it is rapidly oxidized when irradiated in aqueous solution, 74% being destroyed by 75,000 roentgens at a concentration of 50γ per ml. Anderson and Harrison (*1*) have also noticed this sensitivity and have found that plasma does not prevent the reaction. Kung, Gaden, and King (*9*) have shown that ascorbic acid as well as tocopherols are destroyed during sterilization of milk by γ-radiation.

The sulfhydryl-containing compounds have been studied extensively in this connection. It has been repeatedly shown by Barron and coworkers (*2–4*) that sulfhydryl compounds are extremely sensitive to ionizing radiation, being oxidized first reversibly to disulfide and then irreversibly to various decomposition products. Rotheram, Todd, and Whitcher (*15*) have also studied the quantitative aspects of cysteine irradiation, showing that oxidation is the sole effect in aqueous solution.

Table I. Comparison of Spectrophotometric and Titrimetric Values

(Concentration of methyl linoleate emulsion, 3.41×10^{-2} M)

Dosage, R. at 25 R./Min.)	Concn. of Conjugated Diene, Spectrophotometric, $M \times 10^5$	Concn. of Peroxide, Titrimetric, $M \times 10^5$
500	13.7	2.86
1000	25.0	5.64
2000	58.9	13.96

Experimental Procedures and Results

Irradiation of Methyl Linoleate Emulsion Weighed amounts of methyl linoleate were mixed with about one fourth their weight of Tween 80 and emulsified by dropwise addition of water or appropriate buffer until an oil-in-water emulsion formed, when dilution to the desired volume could be accomplished. The methyl linoleate was prepared from tetrabromostearic acid (*10*) and purified by passage through an alumina column and distillation. The ester was stored in small ampoules at 0° C. under carbon dioxide. Material from a freshly opened ampoule had negligible diene conjugation and was used until appreciable

Table II. Effect of Catalase on Irradiation of Methyl Linoleate

(Concentration of methyl linoleate. 3.4 × 10^{-2}M. Dosage, 1000 r. at 25 r./min.)

	Concn. of Peroxide, Titrimetric, M × 10^5	Concn. of Conjugated Diene, Spectrophotometric, M × 10^5	Ionic Yield
No catalase present at time of irradiation	5.90	14.5	72.5
4 × $10^{-10}M$ catalase, added after irradiation	5.30	...	...
4 × $10^{-10}M$ catalase present at time of irradiation	...	16.1	80.5

oxidation had occurred as evidenced by a peak at about 231 mμ, when it was set aside for repurification and a fresh ampoule was opened.

This procedure produced emulsions with particle size of about 5 microns. Particle size evidently has considerable effect on the extent of the reaction, and it was important to prepare the emulsion by a standardized procedure to get comparable results. It was felt that the use of Tween 80 introduced no source of error into the procedure, as under these conditions it was not measurably affected by 1000-roentgen x-irradiation.

Irradiation was carried out as described previously (*11*). The unirradiated and irradiated emulsions were diluted simultaneously with at least threefold volumes of 95% ethyl alcohol, and the solutions thus formed were analyzed spectrophotometrically by comparing their absorbances at 231 mμ. The values thus obtained were converted by appropriate calculations (*11*) to concentrations of conjugated diene and to ionic yields.

In this experiment a correlation was sought between the increase in the concentration of conjugated diene and the peroxide content of the emulsion. For analysis of the latter, a procedure has been devised which gives reproducible results when applied to the system under study. Five milliliters of the aqueous emulsion is dissolved in 5 ml. of *tert*-butyl alcohol in a 250-ml. glass-stoppered flask, 3 drops of glacial acetic acid is added, and the solution is swirled to mix the acid quickly. One-half milliliter of freshly prepared 20% aqueous potassium iodide is added and the solution is stoppered, shaken, and placed in the dark for exactly 15 minutes. At the end of this time, 100 ml. of distilled water is added, and the liberated iodine is titrated with 0.002M sodium thiosulfate. The end point can be determined fairly accurately after some practice, even in the absence of an indicator.

The peroxide values thus obtained may be only relative, but have been found to be of the same order of magnitude as the amounts of conjugated diene. Table I indicates that peroxide values obtained by titration paralleled the spectrophotometric data in all cases and that the two sets of data are quantitatively related.

Effect of Catalase The effect of catalase on the irradiation-induced oxidation of methyl linoleate emulsions was investigated to ascertain whether hydrogen peroxide might be a contributing factor in the reaction, and whether catalase would have any effect on the hydroperoxides formed during the oxidation.

The experimental conditions were identical with those described above, except that in one case catalase (obtained from Vita-Zyme Laboratories, Inc.) at a final concentration of 4 × $10^{-10}M$ was used to prepare the emulsion in place of water. Analyses were performed both spectrophotometrically and titrimetrically. The results, summarized in Table II, indicate that neither catalase nor hydrogen peroxide has any appreciable influence on the formation of hydroperoxides in this reaction.

Effect of Vitamin A The effect of vitamin A on the reaction was studied using emulsions of methyl linoleate and vitamin A acetate purchased from Distillation Products Industries, mixed in various proportions. The method of preparing the emulsions and the conditions for irradiation and analysis were as described, except that the absorption peak at 328 mμ was also recorded as a measure for vitamin A. The results are tabulated in Table III. Three experiments are shown, illustrating the effect of changing the concentration of both solutes.

Evidently vitamin A inhibits the reaction at the highest concentration but is almost completely destroyed at the lowest. In the absence of methyl linoleate, vitamin A is not affected by this amount of irradiation.

Effect of Tocopherol The effect of tocopherol on the reaction was studied using *d*-γ-tocopherol (purchased from Distillation Products Industries). The reaction was carried out as described for vitamin A, except that a measure of tocopherol concentration was obtained from its absorption maximum at 298 mμ, which could be estimated at tocopherol concentrations greater than 3.4 × $10^{-4}M$. Below this minimum concentration the effect of the vitamin on the linoleate oxidation alone was measured. Several experiments were performed in order to determine the minimum protective concentration of the vitamin (Table IV).

Table IV. Effect of *d*-γ-Tocopherol on Irradiation of Methyl Linoleate

(Concentration of methyl linoleate. 2.56 × 10^{-2}M. Dosage. 1000 r. at 25 r./min.)

Concn. of d-γ-Tocopherol, M × 10^5		Concn. of Conjugated Diene, M × 10^5	Ionic Yield
Preirradiation	Postirradiation		
0	...	22.6	113
13.6	...	0	0
68.0	68.0	0	0
340.0	340.0	0	0
0	...	21.0	105
2.69	...	3.23	16.1
8.07	...	0	0
13.5	...	0	0
0	...	16.1	80.5
0.265	...	11.3	56.5
1.32	...	9.68	48.4

The data indicate that *d*-γ-tocopherol inhibits the irradiation-induced oxidation of 2.5 × $10^{-2}M$ methyl linoleate emulsions at all concentrations above

Table III. Effect of Vitamin A on Irradiation of Methyl Linoleate

(Dosage. 1000 r. at 25 r./min.)

Concn. of Vitamin A, M × 10^4,		Concn. of Methyl Linoleate, Preirradiation, M × 10^2	Concn. of Conjugated Diene, Postirradiation, M × 10^5	Ionic Yield from Methyl Linoleate Only
Preirradiation	Postirradiation			
10	10	0.5	0	0
3	2.2	2.3	8.0	40
0.6	0.3	3.0	20	100
3	2.5	2.3	4.8	24
0.6	0.3	2.3	14.5	73
0.3	0.1	2.3	20	100
6.1	6.1	0	...	...
0	0	3.0	25.8	129
6.1	4.9	3.0	0	0

$2.69 \times 10^{-5}M$. At those concentrations at which it could be measured, no destruction of the tocopherol occurred.

Effect of Calciferol The effect of calciferol on the reaction was investigated because compounds of this type have been reported (*16*) to undergo changes when exposed to ionizing radiation. For this reaction emulsions of calciferol (purchased from Sterwin Chemicals, Inc.) and methyl linoleate were mixed in the desired proportions and irradiated in the usual manner. Because the absorption maximum for calciferol is at 265 mμ, it was necessary to devise a means of analysis which would permit the measurement of this maximum in the presence of the conjugated diene peak at 231 mμ.

Table V. Effect of Calciferol on Irradiation of Methyl Linoleate

(Concentration of methyl linoleate. 3.46 × 10^{-2}M. Dosage. 1000 r. at 25 r./min.)

Concn. of Calciferol, M × 10^3		Concn. of Conjugated Diene, M × 10^5	Ionic Yield
Preirradiation	Postirradiation		
0	...	26.6	133
0.73	0.73	12.1	61
1.73	1.73	11.3	57
3.46	3.46	12.1	61

This was done by partition of the mixture between iso-octane and 80% aqueous ethyl alcohol. The calciferol was distributed in favor of the iso-octane (K = 4), while the conjugated diene was found largely in the alcohol layer. Corrections were applied in both cases for material remaining in the other layer. Table V summarizes the results of these determinations.

It appears from these data that calciferol afforded partial protection for the linoleate independent of the concentration of calciferol, within the range studied. The calciferol itself apparently was not affected by the reaction, and, indeed, no detectable change occurred in $8 \times 10^{-4}M$ emulsions of calciferol at dosages as high as 4000 r.

Effect of Ionol The effect of a commercial antioxidant, *Ionol*, as an inhibitor of the reaction was also investigated. As can be seen from Table VI, Ionol prevented the oxidation of linoleate at concentrations of $2.82 \times 10^{-5}M$ and above. In this respect it compares favorably with tocopherol as an antioxidant. Its characteristic absorption maximum (280 mμ, $\epsilon = 1.88 \times 10^3$) was undetectable at the concentrations present in the mixtures.

Effect of Ascorbic Acid The effect of ascorbic acid (purchased from Merck & Co., Inc.) on the reaction was analyzed by a method similar to that employed for calciferol. By partitioning the emulsion between 60% aqueous ethyl alcohol and iso-octane it was possible to measure the ascorbic acid in the aqueous ethyl alcohol by means of its absorption maximum at 265 mμ ($\epsilon = 9.02 \times 10^3$). The conjugated diene was distributed in favor of the iso-octane (K = 3) and could be determined in this solvent by using a correction factor. Because of the extreme instability of ascorbic acid solutions, these were prepared immediately before irradiation and stabilized immediately after irradiation with an equimolecular amount of potassium cyanide. Table VII indicates that ascorbic acid, at concentrations equal to or greater than 9.4 mole %, completely protected the ester from the effects of irradiation. At 5.0 mole %, the vitamin still afforded partial protection. At both concentrations, the vitamin itself was partially destroyed by irradiation.

Effect of Cysteine And Glutathione Cysteine and glutathione (purchased from Nutritional Biochemicals Corp.) were investigated as examples of sulfhydryl-containing compounds which have been used with some success in vivo to minimize the effect of radiation damage. Cysteine had previously been shown (*17*) to inhibit the irradiation-induced autoxidation of linoleic acid. In the present case of methyl linoleate emulsions the reaction was also carried out in slightly alkaline medium (citrate-phosphate buffer at pH 8), as sulfhydryl compounds are known to be more efficient antioxidants at alkaline pH (*8*). The effect of glutathione may be seen in Table VIII, while that of cysteine is shown in Table IX.

Table VI. Effect of Ionol on Irradiation of Methyl Linoleate

(Concentration of methyl linoleate. 2.55 × 10^{-2}M. Dosage. 1000 r. at 25 r./min.)

Concn. of Ionol, M × 10^5	Concn. of Conjugated Diene, M × 10^5	Ionic Yield
0	7.3	36.3
1.41	1.2	6.1
2.82	0	0

Table VII. Effect of Ascorbic Acid on Irradiation of Methyl Linoleate

(Concentration of methyl linoleate. 2.56 × 10^{-2}M. Dosage. 1000 r. at 25 r./min.)

Concn. of Ascorbic Acid, M × 10^3		Concn. of Conjugated Diene, M × 10^5	Ionic Yield
Preirradiation	Postirradiation		
0	...	8.1	40.3
1.35	1.18	5.7	28.3
2.65	2.43	0	0

It is evident that both compounds afford some protection to the methyl linoleate, although at much higher concentrations than in the cases of tocopherol or Ionol. Two interesting observations were made during these experiments. In one case, a sample of linoleate was used which, from the height of its peak at 231 mμ, already was extensively oxidized. Glutathione not only did not protect emulsions of this sample from irradiation damage but actually enhanced the reaction. These emulsions were not buffered and attained a pH of 4 at the end of the irradiations. The authors have speculated that the disulfide probably present in these solutions was responsible for the contrary reaction. Amperometric titrations indicated an extensive oxidation of glutathione in these cases, even before irradiation.

Table VIII. Effect of Glutathione on Irradiation of Methyl Linoleate

(Concentration of methyl linoleate. 2.55 × 10^{-2}M. Dosage. 1000 r. at 25 r./min.)

Concn. of Glutathione, M × 10^4	Concn. of Conjugated Diene, M × 10^5	Ionic Yield
0	18.5	92.5
1.02	6.45	32.3
2.03	8.06	40.3
4.06	4.03	20.2

During the experiments with cysteine, the gradual appearance of an absorption peak at 275 mμ was consistently noticed. This maximum could also be observed in an emulsion containing autoxidized methyl linoleate and cystine, and it is possible that peroxide-catalyzed compound formation takes place between these latter two substances (*7*).

Discussion

It is evident that the irradiation-induced autoxidation of methyl linoleate is markedly influenced by the presence of certain antioxidants in admixture with it. As is to be expected, the lipide-soluble antioxidants were more effective than the water-soluble compounds, tocopherol and Ionol being by far the most efficient antioxidants employed. Destruction of the antioxidant itself occurred in the cases of vitamin A, ascorbic acid, glutathione, and cysteine. Calciferol was not affected, and tocopherol and Ionol were not oxidized by this dose at concentrations high enough for measurement.

In searching for signs of this reaction in the animal body the influence of many of the compounds reported above will have to be considered. With the possible exception of tocopherol, the

Table IX. Effect of Cysteine on Irradiation of Methyl Linoleate

(Concentration of methyl linoleate. 2.55 × 10^{-2}M. Dosage. 1000 r. at 25 r./min.)

Concn. of Cysteine, M × 10^2	Concn. of Conjugated Diene, M × 10^5	Ionic Yield
0	20.2	101
0.11	3.23	16.2
1.01	0	0

concentrations which inhibit the autoxidation in vitro are considerably greater than those which exist in plasma.

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CHEMICAL RESIDUES

Determination of Perchloroethylene in Strawberries

D. A. MAPES and S. A. SHRADER

The Dow Chemical Co., Midland, Mich.

The vapors of perchloroethylene have been found effective in controlling rot-producing mold on strawberries and other fresh fruit. A method is described which involves the extraction of the perchloroethylene from the berries with diethyl ether, followed by an evaporation in the presence of ethylbenzene, and the determination of the chloride by a nephelometric method. Experimental data on known mixtures and on fumigated berries are given.

THE VAPORS OF PERCHLOROETHYLENE have been found effective under certain conditions in controlling rot-producing mold on strawberries and other fresh fruits (*2, 3*). One factor which must be considered before any foodstuff is treated chemically is the hazard that may be associated with residual fungicide. In order to evaluate this problem, it is necessary to establish the quantity of perchloroethylene remaining after treatment. The analytical method described herein is applicable to the determination of small amounts of perchloroethylene in treated strawberries which do not contain other chlorinated organic compounds.

As no sensitive and selective chemical test for perchloroethylene was available, it was found necessary to concentrate the compound by extraction and careful evaporation and to base the analysis on a chlorine determination. The perchloroethylene is extracted from the strawberries with diethyl ether. Ethylbenzene is added to the extract, the ether is removed by evaporation, and the ethylbenzene containing the perchloroethylene is burned in an oxygen bomb. The resulting chloride is determined by a nephelometric procedure.

Apparatus. A Parr oxygen bomb (*1*, No. 1102), with electrical ignition unit, is required for burning the ethyl benzene. The chloride is measured with a photoelectric colorimeter (Lumetron Model 402EM) using 20 × 40 mm. nephelometric cells.

Test tubes, 15 × 150 mm., marked to contain 3 ml., are used for the final evaporation.

Reagents. Oxygen gas (from cylinder); diethyl ether, anhydrous; ethylbenzene, redistilled; alcohol, Formula 30, chloride-free.

Silver nitrate solution. Dissolve 1.7 grams of silver nitrate (reagent grade) in water. Add 12.5 ml. of nitric acid (concentrated C.P.) and dilute to 1 liter with water.

Standard chloride solution. Dissolve 1.6485 grams of dry sodium chloride in water and dilute to 1 liter. Dilute 10 ml. of this solution to 1 liter with water. One milliliter of the final solution contains 10γ of chloride.

Reference solution. Add 2 ml. of standard chloride solution (20γ of chloride) to 23 ml. of water in a 50-ml. volumetric flask, and proceed as directed in the procedure for final test.

Sodium carbonate, 0.5% aqueous solution.

Preparation of Standard Curve

Clean six 50-ml. volumetric flasks with dilute nitric acid and rinse with distilled water. Measure a 0-, 1-, 2-, 3-, 4-, and 5-ml. portion of standard chloride solution into each flask from a microburet. These correspond to 0,