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Antioxidant Effect of Riboflavin in Aqueous Solution

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The antioxidant effects of riboflavin in aqueous solution under irradiation and in the dark were studied. When measurements were made soon after treatment, the linoleic acid hydroperoxide produced increased as the concentration of riboflavin increased. The linoleic acid hydroperoxide produced during light irradiation might arise because of active oxygen produced by the light-sensitizing effect of riboflavin. When the solution was left in the dark after light irradiation and assayed later, the decomposition of hydroperoxide and the recovery at 444 nm tended to increase with higher concentrations of riboflavin. The recovery at 444 nm in the dark might result from a reversible reaction if the reduced was produced by light irradiation to the oxidized form, in the dark. These results suggested that hydroperoxide acted as an electron acceptor in the reversible reaction and that this radical might participate in the reversible reaction from the reduced to the oxidized form, thereby becoming decomposed.

Very fatty foods, which contain much unsaturated fatty acid, in particular, are readily peroxidized by transition-metal ions, active oxygen, and visible or UV light (Agarwal et al., 1984; Kellogg and Fridovich, 1975; Maier and Tappel, 1959; Yamashoji et al., 1979; Thomas et al., 1985; Truby et al., 1987). Consequently, various antioxidants are used to prevent peroxidization of fat in such foods. The most commonly used antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and vitamin E. However, tests with experimental animals suggest that BHA and BHT have adverse effects on the body under some circumstances (Ito et al., 1985; Ponder and Green, 1985; Witschi and Morse, 1985). Vitamin E has no undesirable effects on the body but is unstable to heat (Kajimoto et al., 1987a,b). The antioxidant effect of vitamin E is not of use in very fatty foods that are treated with heat.

In Japan, riboflavin tetrabutyrate is used as a food additive to enrich the vitamin B_2 level. Riboflavin tetrabutyrate has an antioxidant effect on emulsions in the dark (Toyosaki et al., 1987a); the effect is due to the riboflavin itself (Toyosaki et al., 1987b). However, contrary to expectation, riboflavin tetrabutyrate stimulates oxidation under light irradiation. The cause might be the active oxygen produced, which acts in fat peroxidation, or the cause might be the photolysis of riboflavin itself, which did away with its antioxidant effect. Contradictory findings have been reported. Ohama and Yagi (1969) showed that hydroperoxide production is suppressed when a mixture of riboflavin tetrabutyrate and linoleic acid is irradiated with light. Terada (1975) reported that the suppression is due to the riboflavin moiety. The reason for the discrepancy is unknown but may have arisen from differences in the experimental conditions. The purpose of this report is to confirm whether the antioxidant effect of riboflavin occurs even during light irradiation; we assumed, based on the report of Terada (1975) and results of our own group (Toyosaki et al., 1987a,b), that riboflavin has an antioxidant effect. The antioxidant effect of riboflavin being again suggested by our results here, the next step was to identify the mechanism of this effect.

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MATERIALS AND METHODS

Materials. Materials were purchased from the following sources: riboflavin (more than 99% pure), linoleic acid (more than 99% pure), lipoxygenase (from soybean, Type I, lyophilized), and peroxidase (from horseradish, Type X, crystallized) were from Sigma Chemical Co. (St. Louis, MO); Kieselgel 60 G and 60 PF_{254} were from Merck (Darmstadt, FRG); the 0.25-mm thin-layer chromatography (TLC) was done with use of a TLC apparatus from Yazawa Scientific Apparatus Mfg. Co., Ltd. (Tokyo, Japan); nitro blue tetrazolium (NBT), *N',N'*-dimethylformamide, and *o*-dianisidine were from Tokyo Kasei Co., Ltd. (Tokyo, Japan); all other reagents were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Preparation of Linoleic Acid Hydroperoxide. Linoleic acid hydroperoxide was prepared by the procedure of Matsuda et al. (1978) by enzymatic peroxidation with lipoxygenase. The standard reaction mixture containing 32 mM linoleic acid, 0.1% Tween 80, 50 mM Na_2HPO_4/NaOH buffer (pH 9.0), and 50 units of the lipoxygenase in a total volume of 20 mL was incubated in a conical flask to facilitate flushing with pure oxygen. The reaction mixture was stirred mechanically for 40 min under a stream of pure oxygen. After incubation, hydroperoxide was extracted with diethyl ether. The formation of linoleic acid hydroperoxide was monitored by measurement of the increase in the absorbance at 234 nm. The hydroperoxide was purified by TLC with *n*-hexane/diethyl ether/acetic acid (60:40:1, v/v/v) as the solvent system and monitored under UV light.

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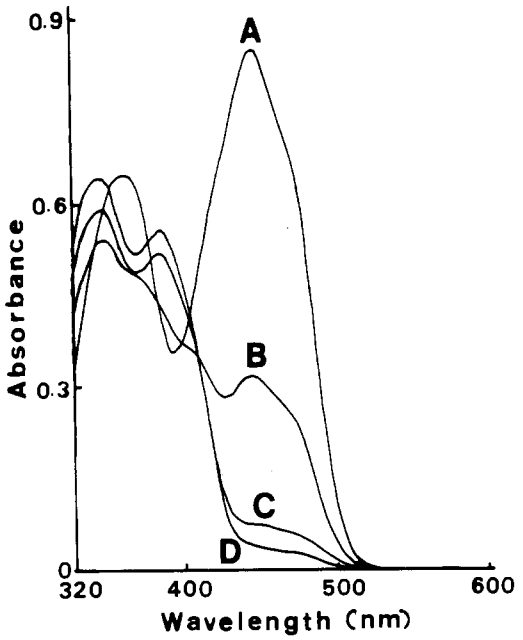

Figure 1. Absorption spectra of riboflavin with linoleic acid after light irradiation: A, no irradiation; B, irradiation for 30 min; C, irradiation for 60 min; D, irradiation for 240 min. The light intensity was 11 000 lx.

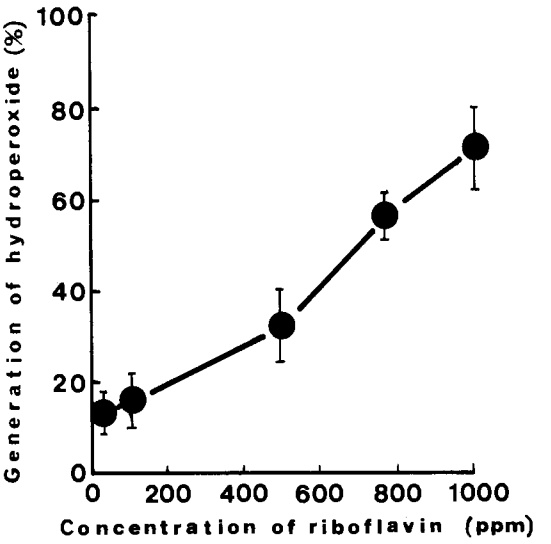

Figure 2. Effect of the concentration of riboflavin on the generation of linoleic acid hydroperoxide with light irradiation. Irradiation was for 120 min at 11 000 lx. Results are given as the percentage compared to the 100% saturated solution of linoleic acid hydroperoxide referred to as the standard reaction mixture in Materials and Methods. Conditions: 100% hydroperoxide, 200 μM peroxide. Each point represents the mean of four trials; the standard deviation is given.

Preparation of Sample and Irradiation Conditions. The reaction mixture contained known amounts of riboflavin, known amounts of linoleic acid or hydroperoxide, 40 mM phosphate buffer (pH 7.4), and 10% ethanol. The total reaction volume was 5.0 mL, and the mixture was stirred during irradiation at 450 nm at 20 °C for up to 60 min.

Measurement of Hydroperoxide. The concentration of linoleic acid hydroperoxide was assayed by the absorbance at 480 nm by the method of Mitsuda et al. (1966).

Assay of Superoxide Anion and Hydrogen Peroxide. Superoxide anion (O_2^-) was measured by the NBT reduction method (Korycka-Dahl and Richardson, 1978). The precipitate of diformazan produced upon reduction of NBT by O_2^- was obtained by centrifugation at 3000 rpm for 10 min and dissolved in 4.0 mL of *N',N'*-dimethylformamide. Absorbance was measured at 560 nm to estimate the amount of O_2^- produced in the test sample from a calibration curve prepared with the use of a standard diformazan solution. Hydrogen peroxide (H_2O_2) was measured by the enzymatic reduction of the peroxide with the *o*-dianisidine/peroxidase system as described previously (Fontes et al., 1981); the absorbance of the complex so formed was measured at 440 nm with a 200-10 spectrophotometer (Hitachi, Ltd., Tokyo, Japan).

RESULTS AND DISCUSSION

Figure 1 shows the absorption spectra of a mixture of 40 mM phosphate buffer (pH 7.4, containing 10% ethanol) with riboflavin (1000 ppm) and linoleic acid (10 mM) measured at set times after light at the intensity of 11 000 lx. Riboflavin gives rise to two absorption peaks at 356 and 444 nm, the latter of which is due to its isoalloxazine ring. The peak at 444 nm decreased after the irradiation. However, the peak tended to recover when the irradiation ended and the solution was left in the dark, with maximum recovery to about 84% (data not shown). Consequently, some 16% of the riboflavin seemed to have decomposed. When the irradiation continued for 240 min or more, recovery was 0% and the spectrum probably was that of the reduced form of riboflavin. The recovery of the decrease in the peak at 444 nm caused by light irradiation was almost constant regardless of the concentrations of the riboflavin (0–1000 ppm).

Ohama and Yagi (1969) reported that riboflavin tetrabutyrate is decomposed in the reaction by which hydroperoxide is formed from linoleic acid and that linoleic acid hydroperoxide is formed at the same time. Terada (1975) reported that the decomposition of the isoalloxazine ring in riboflavin causes the decomposition of hydroperoxide at the same time. Hydroperoxide is an index of oxidation, and low levels are consistent with the presence of an antioxidant compound. Consequently, the change in the absorption of isoalloxazine is one possible index of riboflavin having an antioxidant effect.

The mixed system of riboflavin at various concentrations in a solution of linoleic acid at a fixed concentration was irradiated with 11 000 lx of light. The amount of hydroperoxide produced was compared with the amount of hydroperoxide decomposed when the solution was left after the light irradiation. After the irradiation, the amount of hydroperoxide generated tended to increase with higher concentrations of riboflavin (Figure 2). The results were similar to those obtained with an emulsion system (Toyosaki et al., 1987a). However, when the solution was not irradiated, the decomposition of hydroperoxide and the recovery at 444 nm tended to increase with higher concentrations of riboflavin (Figure 3). These results suggested that the changes in the recovery at 444 nm were influenced by the concentration of hydroperoxide present. That is, the amount of hydroperoxide increased during light irradiation with higher concentrations of riboflavin, and the decomposition of hydroperoxide increased with higher concentrations of riboflavin when the solution was left in the dark after irradiation; there might be some relationship between the recovery at 444 nm and hydroperoxide. The recovery at 444 nm in the dark seemed to involve a reversible reaction from the reduced to the oxidized form of riboflavin; there must be an electron acceptor in the system. It is likely that the hydroperoxide produced acted as an electron acceptor in this system.

The effects of the irradiation intensity on the amount of hydroperoxide generated and the recovery at 444 nm

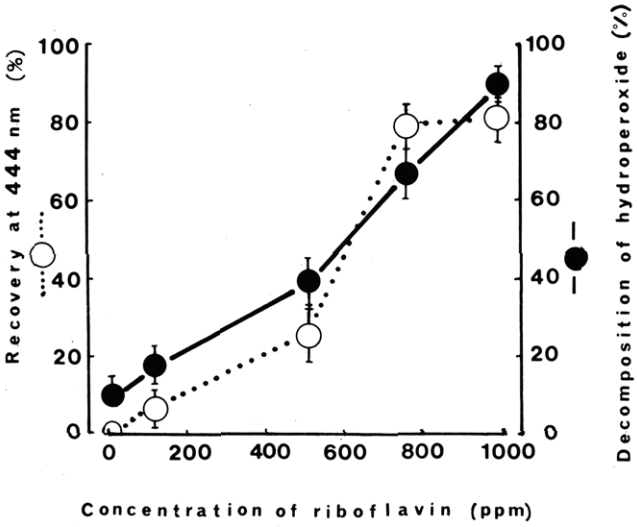

Figure 3. Effect of the concentration of riboflavin on the decomposition of linoleic acid hydroperoxide and recovery at 444 nm in the dark. Incubation in the dark was for 120 min. Results for recovery at 444 nm are expressed as a percentage, with the peak at 444 nm after 240 min of irradiation at 11 000 lx as 0% and the peak without irradiation as 100%. Each point represents the mean of four trials; the standard deviation is given.

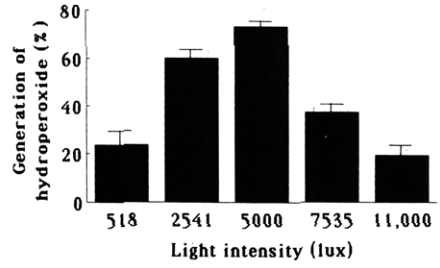

Figure 4. Effects of light intensity on the generation of linoleic acid hydroperoxide. Irradiation time was 120 min. Each column shows the mean of four trials; the standard deviation is given.

were investigated. Here, the concentrations of riboflavin and linoleic acid were kept constant. The amount of hydroperoxide generated was maximum at the irradiation intensity of 5000 1x and decreased with greater intensities (Figure 4). The decrease in the peak at 444 nm caused by light irradiation was almost constant with different light intensities (data not shown). When the solution was left in the dark immediately after the irradiation for a set time, the decomposition of hydroperoxide and the recovery from the decrease at 444 nm declined as irradiation intensities increased from 518 to 5000 1x; however, both ratios increased with irradition intensities of 7535 1x or more (Figure 5).

Generally speaking, flavins have a light-sensitizing effect and produce active oxygen during light irradiation. The amounts of O_2^- produced from riboflavin seem to be affected by the light intensity (Table I), with a maximum somewhere around 5000 1x; the seeming decrease at higher light intensities may arise because the reaction is too rapid to monitor. Consequently, the increased production seen in Figure 4 of hydroperoxide caused by light seemed to result, when the intensity was relatively weak along the range of light intensities we used, from a lipid peroxidation reaction with active oxygen produced. Whether active oxygen was produced or not in this reaction was our next question.

Table I also shows the amount of H_2O_2 produced. H_2O_2 was maximum at 5000 1x. When the solution was left in the dark, active oxygen was not produced. Consequently,

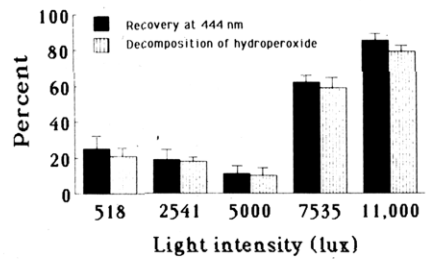

Figure 5. Effect of light intensity on the rate of the decomposition of linoleic acid hydroperoxide and recovery in the decrease in the peak at 444 nm in the dark after light irradiation. Light irradiation was for 120 min. Incubation in the dark was for 120 min. Each column represents the mean of four trials; the standard deviation is given.

Table I. Generation of Superoxide Anion and Hydrogen Peroxide

	O_2^-,[a] μM/min		H_2O_2,[a] nM/min	
light, lx	light[b]	dark[c]	light[b]	dark[c]
518	3.5	0	4.9	0
2541	9.9	0	10.2	0
5000	24.3	0	26.0	0
7535	1.2	0	2.4	0
11 000	0.05	0	0.08	0

[a] Key: O_2^-, superoxide anion; H_2O_2, hydrogen peroxide. Means of duplicate samples. [b] Irradiation for 60 min. [c] Kept in the dark for 120 min.

the increase in hydroperoxide caused by increasing light irradiation from 518 to 5000 1x probably arose from the stimulation by active oxygen of the lipid peroxidation reaction. The reason for the increased recovery at 444 nm with the increased irradiation intensity when the solution was left in the dark was that the amount of active oxygen produced was small and caused little decomposition of riboflavin. When the irradiation intensity is weak, the active oxygen produced stimulates the decomposition of riboflavin (Toyosaki et al., 1983; see Table I). Thus, it seemed likely that riboflavin was changed in a reversible reaction from its reduced to its oxidized form in the dark.

Next, we wanted to know whether hydroperoxide was involved in the reversible reaction when the solution was left in the dark. Riboflavin solution (1000 ppm) was irradiated by light at 11 000 1x for 120 min, and then various amounts of hydroperoxide were added to give certain final concentrations. The mixture was left for 120 min in the dark, and the recovery at 444 nm and the decomposition of hydroperoxide were measured (Figure 6). The recovery at 444 nm tended to increase with higher concentrations of hydroperoxide. Thus, the recovery at 444 nm depended on the amount of hydroperoxide present. The decomposition of hydroperoxide also tended to increase with higher concentrations of hydroperoxide. The results suggested that hydroperoxide was probably involved in the reversible reaction as an electron acceptor. However, it was not clear whether the hydroperoxide itself was decomposed in the reaction in which it acted as an electron acceptor; other decomposition mechanisms are also possible. The results showed that the reversible reaction proceeded as long as hydroperoxide existed and that at the same time hydroperoxide was decomposing.

Our results are opposite to those of Ohama and Yagi (1969), who reported that when riboflavin tetrabutyrate is irradiated by light in the presence of linoleic acid, the production of hydroperoxide is suppressed. They suggested that the mechanism involves an irreversible reaction that occurs during the irradiation. They concluded that the decomposition of hydroperoxide accompanies the de-

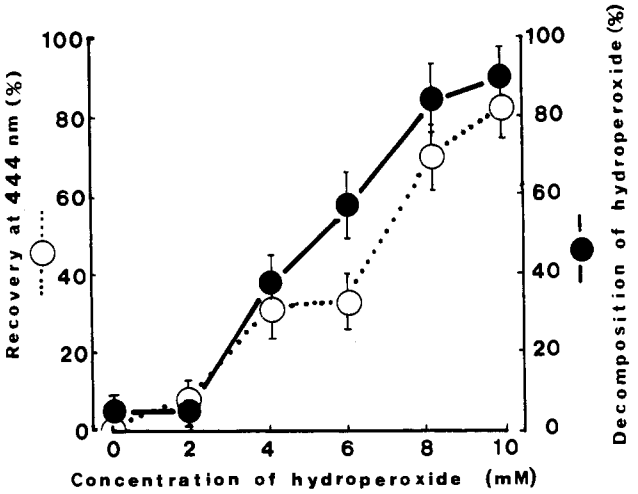

Figure 6. Effects of the concentration of linoleic acid hydroperoxide on the rate of decomposition of hydroperoxide and recovery at 444 nm in the dark. Reaction conditions were as follows: A riboflavin solution (1000 ppm) was irradiated at 11 000 lx for 120 min. After irradiation, different amounts of linoleic acid hydroperoxide were added to the riboflavin solution and the resultant mixtures stirred during incubation in the dark for 120 min. Each point represents the mean of four trials; the standard deviation is given.

composition of riboflavin. Terada (1975) also reported that hydroperoxide decomposes as the isoalloxazine ring of riboflavin is decomposed. His results suggested that riboflavin stimulates oxidation rather than acting as an antioxidant. He did not confirm the presence of active oxygen. If the isoalloxazine ring of riboflavin is decomposed, riboflavin can be assumed to have been decomposed. We found that riboflavin caused lipid peroxidation during irradiation by light; the hydroperoxide produced during the irradiation acted as an electron acceptor when the solution was left in the dark, and this hydroperoxide was decomposed at the same time. We think of these phenomena as being equivalent to antioxidant activity. Thus, this antioxidant has a new kind of mechanism. Riboflavin could be used as a potent antioxidant in foodstuffs that need heat processing.

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Registry No. Riboflavin, 83-88-5; linoleic acid, 60-33-3; linoleic acid hydroperoxide, 25657-09-4.

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