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Role of Lipid in Sulfite-dependent Propofol Dimerization

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Background: **During long-term intravenous infusions, sulfite in sulfite-containing propofol emulsions can cause the peroxidation of lipid and dimerization of propofol. This study evaluated the role of lipid in sulfite-dependent propofol dimerization by determining the effects of individual fatty acids in soybean oil emulsion and peroxidized lipids in a model system.**

Methods: **Individual fatty acids, stearic (18:0), oleic (18:1), linoleic (18:2), linolenic (18:3), and arachidonic (20:4), were added to sulfite-containing propofol emulsion and incubated for 90 min at 37°C. Model systems containing soybean oil (100 μl), water (900 μl), propofol (10 mg/ml), and sulfite (0.25 mg/ml) composed of oils with different peroxide values were allowed to react for 60 min at room temperature. After the reactions, propofol dimer and propofol dimer quinone were analyzed by reversed-phase high-pressure liquid chromatography.**

Results: **Propofol did not dimerize when added to aqueous sulfite unless soybean oil was also included. The addition of the polyunsaturated fatty acids (linoleic, linolenic, arachidonic) to sulfite-containing propofol emulsion resulted in large increases of propofol dimerization compared with stearic or oleic acid. Using biphasic mixtures of soybean oil and aqueous sulfite, propofol dimerization increased with increasing peroxide content of the oil. In propofol emulsion, lipoxidase and ferrous iron in the absence of sulfite also caused the dimerization of propofol.**

Conclusions: **These results show that lipid can play a significant role in sulfite-dependent propofol dimerization. The relation of dimerization to polyunsaturated fatty acid and soybean oil peroxide content suggests that sulfite reacts with unsaturated lipid or peroxide-modified lipid to facilitate propofol dimerization.**

THE inorganic compound sulfite (SO_3^{2-}) is added to a number of aqueous drug preparations as an antioxidant and a preservative.[1–3] It is commonly added as the potassium or sodium salts of bisulfite (HSO_3^-) or metabisulfite ($S_2O_5^{2-}$), in quantities of from 0.15 to 1.0 mg/ml drug solution. The sulfite ion from these compounds is protective to drug formulations in part by chemically reducing oxygen, thus serving as an oxygen scavenger.[4] In addition to these actions, it is added to a propofol emulsion as sodium metabisulfite (0.25 mg/ml) to inhibit microbe growth.[5]

Under certain circumstances, sulfite can promote, rather than inhibit, the oxidation of some compounds. Sulfite has been shown to cause the peroxidation of lipids in corn oil,[6] soybean oil,[7] and fatty acid.[8,9] Sulfite has also been shown to oxidize the coumarin derivative scopoletin under various conditions, including in the presence of hydrogen peroxide (H_2O_2) and horseradish peroxidase, in the presence of hydrogen peroxide and xanthine oxidase, and in the presence of *tert*-butylhydroperoxide.[10] It is believed that the one-electron oxidation of sulfite to the sulfite anion radical is the initial event in sulfite-dependent oxidant reactions. The biphasic effect of sulfite as an antioxidant *versus* pro-oxidant is thought to be related to sulfite concentrations in which lower sulfite favors pro-oxidation.[10]

More recently, sulfite in a commercial propofol emulsion for intravenous injection has been shown to be responsible for two concurrent oxidant processes, the peroxidation of emulsion lipids[7] and the oxidation of propofol.[11,12] This propofol oxidation involves the coupling of two propofol molecules to yield two major propofol dimer products, one of which, a quinone, causes emulsion yellowing. Both processes seem to occur to a limited extent in unopened vials, but they increase in time after opening, such as during an intravenous infusion.[7,12]

Mechanisms that lead to sulfite-dependent oxidation of propofol in lipid emulsions are of interest because this process represents an unwanted reaction of sulfite with the drug substance generating a potentially detrimental quinone-type compound.[13] Lipids have not been considered as participating in these reactions. However, Brestel *et al.*[14] showed that sulfite can interact with peroxidized lipid resulting in sulfite depletion and consumption of oxygen. Because soybean oil emulsions contain unsaturated lipids[15] that are susceptible to peroxidation and free radical chemistry,[16] a role of lipid in sulfite-dependent propofol oxidation was investigated. This was done by evaluating sulfite-dependent propofol dimerization in soybean oil emulsions in the presence of various fatty acids and in model systems containing soybean oil of varying lipid peroxide contents.

Materials and Methods

Chemicals

Pure soybean oil (100%) containing no preservatives (Procter and Gamble, Cincinnati, OH) was periodically purchased from local sources. The oils (five total) were stored for various periods of time under nitrogen, air, or 100% oxygen atmospheres. Exposure to oxygen either as air or pure oxygen allowed natural peroxidation of the soybean oil to occur. The peroxide value of each oil was measured by the ferrous-thiocyanate method[17] using *t*-butylhydroperoxide as standard. Soybean oil emulsion (10%) was purchased from Fresenius-Kabi Clayton, LP (Clayton, NC). Propofol (2,6-diisopropylphenol) was

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purchased from Aldrich Chemical Co. (Milwaukee, WI). Sodium metabisulfite, lipoxidase (type I-B, 100,000 U/mg), sodium ascorbate, desferrioxamine, ferrous sulfate, and sodium stearate, oleate, linoleate, linolenate, and arachidonate were obtained from Sigma (St. Louis, MO). Pure propofol dimer and propofol dimer quinone standards were supplied by AstraZeneca Pharmaceuticals LP (Wilmington, DE). One percent propofol emulsion containing sodium metabisulfite was obtained from Baxter Healthcare Corp., Inc. (Deerfield, IL).

Emulsion Reactions

One milliliter soybean oil emulsion, 10%, and 10 mg propofol were added to 20-ml glass vials with screw caps. To each was added various components, including sulfite (0.25 mg/ml, 1.3 mM), ascorbic acid (0.63 mM), lipoxidase (1 mg/ml), and/or ferrous iron (as ferrous sulfate, 1.3 mM). In those reactions to which metabisulfite, lipoxidase, or ferrous sulfate was added, these components were added last to initiate the reaction. Sodium metabisulfite solution was prepared by solubilizing 10 mg sodium metabisulfite in 1 ml deionized water immediately before use. The mixtures were incubated while shaking at 37°C for up to 90 min. After the reaction period, 100 μl NaCl, 10%, was added to each to crack the emulsion. One milliliter ethyl acetate was then added, and the mixtures were shaken. The ethyl acetate phases were removed and stored in the freezer until analysis.

In some experiments, stearic (18:0), oleic (18:1), linoleic (18:2), linolenic (18:3), or arachidonic acids (20:4) as the sodium salts were added to sulfite-containing propofol emulsion. Propofol emulsion (1 ml) was taken from intact commercially prepared vials. After fatty acid addition, the samples were vortexed and incubated for 90 min. After reaction, the samples were cracked, extracted, and analyzed as described below.

Model System Reactions

Soybean oil (100 μl) or sodium linoleate, and propofol (10 mg) were added to 900 μl deionized water or 25 mM sodium phosphate buffer in 20-ml glass vials. To some reactions, desferrioxamine (200 μM) was added. The mixtures were shaken. Sodium metabisulfite (0.25 mg) in aqueous solution was included as the final addition, and the vials again shaken to start the reaction. The reactions were allowed to continue at room temperature for 60 min. After the reaction period, 1 ml ethyl acetate was added, and the vials were shaken to extract propofol and propofol dimerization products into the ethyl acetate phase. The ethyl acetate phases were removed and placed in the freezer until analysis.

Linoleate Reduction

The reduction of linoleate to remove peroxides was done using sodium borohydride ($NaBH_4$) as described by Thiemt and Spiteller.[18] This method entailed the addition of 500 mg $NaBH_4$ to 500 mg linoleate in 100 ml distilled water. The mixture was allowed to stir at room temperature for 1 h. The reaction was stopped by adding 1 N HCl until bubbling ceased to destroy the $NaBH_4$. The linoleic acid was extracted with ethyl acetate and dried under a vacuum. Linoleic acid for control was treated the same, except the addition of $NaBH_4$ was omitted.

Propofol Product Analysis

The ethyl acetate extracts were analyzed on a Beckman System Gold (Beckman Instruments, Fullerton, CA), which had a Solvent Module 126, a Diode Array Detector Module 168, and a manual injector. This instrument was equipped with a Hypersil ODS 150 × 4.6 mm, 5-μm particle size (Supelco Inc., Bellefonte, PA). Propofol and propofol product separation involved the use of a mobile phase gradient system. The initial mobile phase was methanol–5 mM ammonium acetate (70/30, vol/vol), which was run at a flow rate of 0.75 ml/min for 9 min. This was followed by an increase in flow rate to 1.25 ml/min and change to methanol (100%) for 3 min and an isocratic period of methanol–5 mM ammonium acetate (70:30) for 3 min at a flow rate of 0.75 ml/min. A sample size of 50 μl was injected. Propofol dimer was quantitated by its absorbance at 265 nm, and propofol dimer quinone was quantitated by its absorbance at 422 nm. Standard curves were constructed using authentic propofol dimer and propofol dimer quinone dissolved in ethyl acetate. Calculated extinction coefficients (M^{-1} cm^{-1}) for propofol dimer (265 nm) and propofol dimer quinone (422 nm) in ethyl acetate were 22 and 71, respectively.

Statistics

Data were analyzed with Kruskal-Wallis test for multiple comparisons using the Bonferroni adjustments and were considered significant when P was less than 0.05.

Results

Sodium metabisulfite at a concentration of 0.25 mg/ml (1.3 mM) in solution or in 10% soybean oil emulsion was used in the study. This is the concentration and form of sulfite added to propofol emulsions as an antimicrobial preservative (propofol injectable emulsion, 1%, prescribing information; Baxter Healthcare Corp., Inc.), and it is representative of the concentration added to many other drug preparations.[1] The effects of sulfite on propofol dimerization in aqueous solution and in 10% soybean oil emulsion are shown in figure 1. Aqueous sulfite alone did not cause the dimerization of propofol; however, sulfite in soybean oil emulsion did cause the formation of significant quantities of both propofol dimer and propofol dimer quinone. The addition of a small amount of ascorbic acid (0.125 mg/ml, 0.63 mM) to the sulfite-

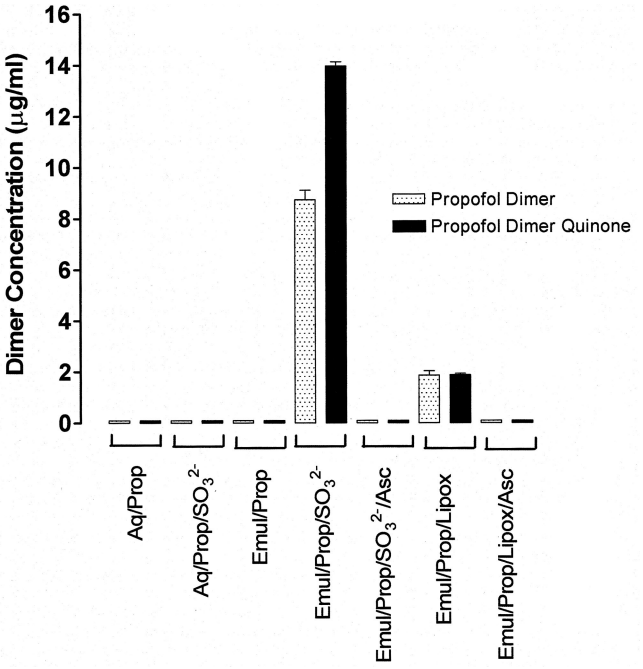


Fig. 1. Propofol dimer formation in aqueous and emulsion systems. Where indicated, the compositions were sodium metabisulfite (SO_3^{2-}), 0.25 mg/ml; ascorbate (Asc), 0.63 mM; and lipoxidase (Lipox), 1 mg. Reactions were performed as described in Materials and Methods. Values represent the mean (± SD) of triplicate reactions. Aq = aqueous; Emul = emulsion; Prop = propofol. *Dotted bars* = propofol dimer; *solid bars* = propofol dimer quinone; *valueless bars*, < 1 μg/ml.

containing emulsions before reaction completely inhibited propofol dimerization.

The addition of lipoxidase (1 mg), an enzyme that catalyzes lipid peroxidation, was found to cause the dimerization of propofol in the absence of sulfite (fig. 1). It was initially attempted to determine the effect of lipoxidase-generated lipid peroxides on sulfite–propofol dimerization in soybean oil emulsions; however, it was found that lipoxidase alone during the incubation period caused propofol dimerization. Similar to sulfite-dependent propofol dimerization, ascorbic acid effectively inhibited lipoxidase-dependent propofol dimerization. The pH of these emulsions after addition of all components ranged from 6.5 to 7.5.

To determine the influence of specific fatty acids on sulfite-dependent propofol dimerization, commercial sulfite-containing propofol emulsions were enriched with individual fatty acids, and propofol dimer formation evaluated after reaction (fig. 2). The polyunsaturated fatty acids linoleic, linolenic, and arachidonic acids each caused large increases in propofol dimerization over that in propofol emulsions to which stearic or oleic acid were added. Although only trace levels of the two propofol dimers were detected in emulsion containing stearic acid, oleic acid-containing emulsion yielded low but significant quantities. Of the polyunsaturated fatty acids, linoleic acid was most effective in increasing propofol dimerization. It increased total propofol dimers approximately 29-fold over that in oleic acid–containing emulsions, followed by arachidonic (15-fold increase) and linolenic acids (20-fold increase). The reactions containing polyunsaturated acids were rapid and noted to occur in less than 30 min at room temperature.

Biphasic mixtures of water (900 μl), soybean oil (100 mg/ml), and propofol (10 mg/ml), each component being at the same concentration as in commercial propofol emulsions,[5] were found to cause rapid propofol dimerization at room temperature when sodium metabisulfite (0.25 mg/ml, 1.3 mM) was added. Only trace concentrations (< 1 μg/ml) were detected without sulfite addition. The emulsifier (lecithin) was not needed for this propofol dimerization to occur and was not added. The abilities of five soybean oils with different peroxide values to facilitate propofol dimerization when sulfite was added are shown in figure 3. The peroxide contents (mmol/ml) of each oil (mean ± SD of triplicate determinations) were 0.08 ± 0.12, 0.12 ± 0.04, 0.21 ± 0.32, 0.92 ± 0.35, and 11.66 ± 3.24. The results show that the quantities of dimers produced increased with soybean oil of increasing peroxide contents. Differences in lipids of lower peroxide values seemed to have greater effects in stimulation propofol dimerization.

To clarify a potential role of lipid peroxides in sulfite-dependent propofol dimerization, model reaction systems containing linoleic acid as the sole lipid were performed. Pure linoleic acid, air exposed for 5 days, was very effective in facilitating propofol dimerization. Consequently, the reactions were performed for only 30 min at room temperature. These reactions were pH buffered to 5.4, midway of the pH range specified on the com-

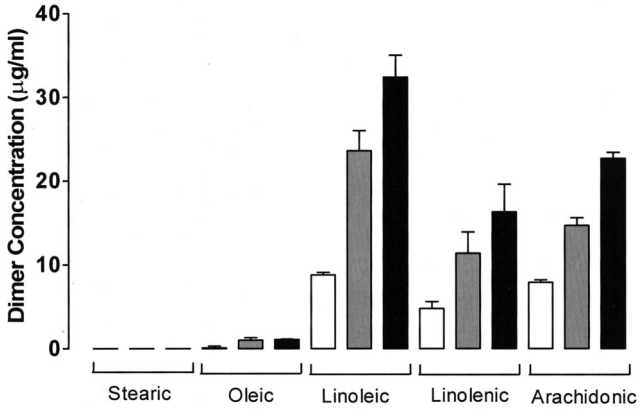


Fig. 2. Effects of fatty acid addition on propofol dimerization in sulfite-containing propofol emulsion. Reactions were performed as described in Materials and Methods. The fatty acid salts added per milliliter emulsion were 16.3 μmol stearic, 16.4 μmol oleic, 16.5 μmol linoleic, 16.7 μmol linolenic, and 15.3 μmol arachidonic. Values represent the mean (± SD) of triplicate determinations. *White bars*, propofol dimer; *gray bars*, propofol dimer quinone; *black bars*, total dimers, *valueless bars*, < 1 μg/ml. All values are significantly different from corresponding values of each other unsaturated fatty acid ($P < 0.05$).

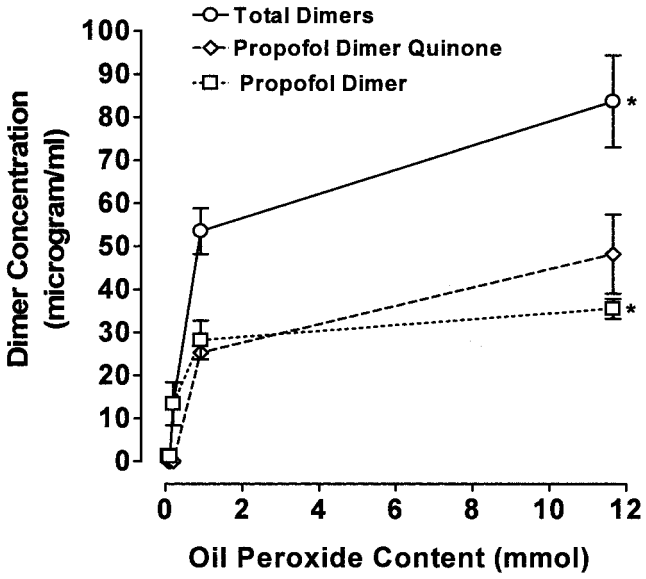


Fig. 3. Propofol dimerization in soybean oil mixtures containing soybean oils of different lipid peroxide contents. Reactions were performed as described in Materials and Methods. Values represent the mean (± SD) of triplicate reactions. * Significantly different from lowest peroxide value in each group ($P < 0.05$).

mercial sulfite–propofol emulsion, because of the acid treatment of the linoleic acid in the reduction process. As shown in figure 4, previous reduction of linoleic acid with $NaBH_4$ effectively inhibited propofol dimerization to both propofol dimer and propofol dimer quinone. The use of similarly air-exposed stearic acid not treated with $NaBH_4$ yielded no detectable propofol dimerization in similar reactions (data not shown).

Because sulfite can act as a reductant,[19] the effect of ferrous ion, which can also function as a simple reductant, on propofol dimerization in 10% soybean oil emulsions containing 1% propofol was examined. When iron was added as ferrous sulfate (1.3 mM), this addition caused a high degree of propofol dimerization and extensive yellowing. The dimerized products were predominately in the form of propofol dimer quinone. After reaction, the dimer values (μg/ml ± SD) were as follows: without $FeSO_4$: propofol dimer, < 1, propofol dimer quinone, 1.3 ± 0.6; with $FeSO_4$: propofol dimer, 10.8 ± 4.7, propofol dimer quinone, 350.3 ± 26.6. While these reactions were incubated at 37°C for 90 min, the reaction also proceeded rapidly at room temperature.

The effect of the trace metal chelator, desferrioxamine, in peroxidized soybean oil–containing reactions was evaluated by adding 200 μM desferrioxamine to the reaction mixtures before reaction. These reactions were performed for 90 min at 37°C at a pH of 5.4. Under these reaction conditions, desferrioxamine exerted a stimulatory effect. The propofol dimer values (μg/ml ± SD, triplicate determinations) obtained were as follows: no desferrioxamine: propofol dimer, 10.3 ± 1.1, propofol dimer quinone, 20.9 ± 2.6; desferrioxamine: propofol dimer, 14.3 ± 1.6, propofol dimer quinone, 34.2 ± 1.7.

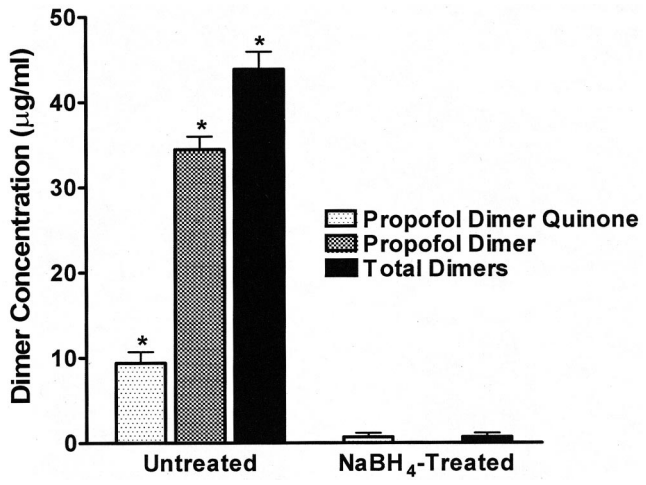


Fig. 4. The effect of $NaBH_4$ linoleic acid reduction on propofol dimerization. The reactions were performed for 30 min at room temperature at a pH of 5.4 as described in Materials and Methods. * Significantly different from $NaBH_4$-treated linoleic acid ($P < 0.05$).

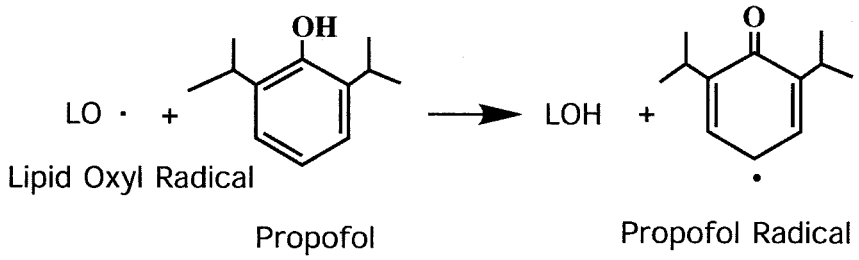
Discussion

This study shows that lipids can play a significant role in sulfite-dependent propofol dimerization. This is shown by the findings that (1) aqueous sulfite did not cause propofol oxidation in the absence of soybean oil emulsion or soybean oil; (2) individual fatty acids when added to sulfite-containing propofol emulsion facilitated propofol dimerization, but to different degrees; and (3) soybean oils of increasing peroxide contents resulted in increased propofol dimerization in the presence of sulfite.

Although sulfite is an antioxidant, the paradoxical effect of sulfite to cause oxidative reactions is due to its reductive properties in the presence of oxygen. Aqueous sulfite on exposure to oxygen oxidizes *via* one- and two-electron removals from the sulfite anion.[20] Abstraction of one electron results in the formation of a sulfite anion radical

$$(SO_3^{2-} \rightarrow SO_3^{\bullet -} + e^{-1}),$$

which is capable of undergoing a number of radical reactions. We have shown,[7] as have others,[8,9] that sulfite peroxidizes lipids, which is a free radical process.[21] Furthermore, electron paramagnetic resonance analysis has directly shown the presence of the sulfite radical in sulfite-containing propofol emulsions.[12] Given the radical chemistry of sulfite, lipid peroxidation may occur by



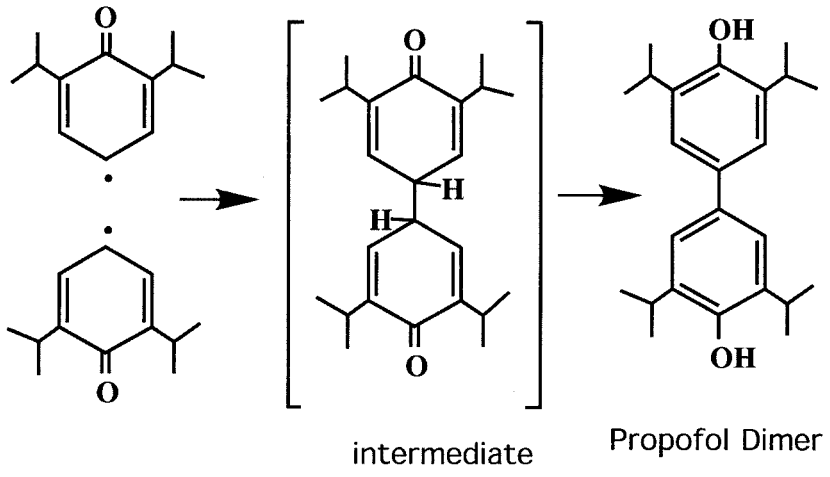


Fig. 5. Scheme for the formation of propofol radical and propofol dimer by a lipid radical.

several processes: (1) direct interaction of sulfite radical with lipid to form lipid radicals

$$(SO_3^{\bullet -} + LH \rightarrow L^{\bullet}, L = \text{lipid});$$

(2) formation of sulfite peroxyl or sulfate radicals

$$(SO_3^{\bullet -} + O_2 \rightarrow {}^{\bullet}OOSO_3^{-})$$

or

$$SO_4^{\bullet -}$$

that interact with lipids; or (3) less likely, the formation of reactive oxygen forms, superoxide or hydroxyl radicals

$$(O_2^{\bullet -} \text{ or } {}^{\bullet}OH)$$

that create lipid radicals.[19]

Propofol dimerization is a process that is initiated by the loss of an electron from the parent molecule. This event, an oxidation of propofol, results in propofol radical formation. A consequence of propofol radical formation is that two propofol molecules can couple to create a propofol dimer, and the propofol dimer can further oxidize to a propofol dimer quinone.[12] Therefore, propofol dimerization can be considered to represent the functioning of propofol as an antioxidant, *i.e.*, electron donor.

The effect of lipid to increase propofol dimerization indicates that a reaction involving the three substances, sulfite, lipid, and propofol, occurs. Using small added quantities of free fatty acids to sulfite-containing propofol emulsions, lipid unsaturation is found to be a major factor in propofol dimerization. Stearic acid, which has no unsaturation, did not result in propofol dimerization. Oleic acid, which has a single unsaturation, resulted in low dimerization. Linoleic, linolenic, and arachidonic acids, which are polyunsaturated lipids, were much more effective. The use of different soybean oils, in which the original fatty acid contents are the same but differ in peroxide contents, furthermore shows that lipid peroxides roughly correlate with the ability of the oil to facilitate sulfite-dependent propofol dimerization. This, coupled with the finding that $NaBH_4$ pretreatment renders linoleic acid much less effective in facilitating propofol dimerization, suggests that lipid peroxides derived from unsaturated lipids may play a role in dimerization. The reason that linoleic acid is most effective when added to sulfite propofol emulsion even though it is less unsaturated than linolenic and arachidonic acids is not apparent. It cannot be ruled out that differences in physical factors that result from fatty acid addition do not play some role.

A scenario of lipid participation in sulfite oxidation relates to initiation and propagation of sulfite radical formation. Formation of the sulfite radical from sulfite requires a process to initiate radical formation (initial electron withdrawal), and propagation reactions involve continuous radical generation. Initiation reactions have been shown to be photolytic,[22,23] metal catalyzed,[24] or

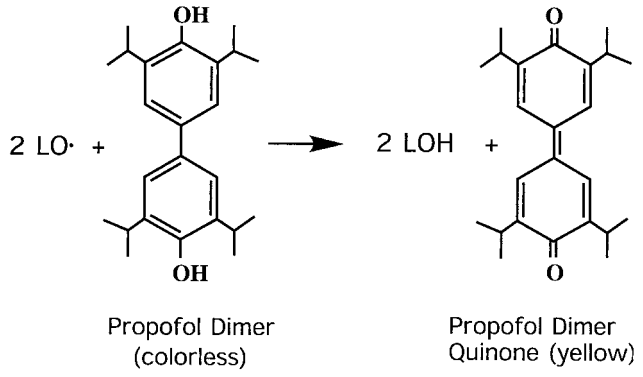


Fig. 6. Scheme for the formation of propofol dimer quinone from propofol dimer by a lipid radical.

performed by enzymes including catalases and peroxidases in the presence of a peroxide.[10,20,25] Brestel *et al.*[14] demonstrated an apparent ability of 15-hydroperoxyeicosatetraenoic acid to play this role. In view of the ability of sulfite to react with peroxides in the presence of the various cofactors, it is hypothesized that sulfite interacts with lipid peroxides *via* the mechanism below and outlined previously[14] to cause sulfite radical formation:

$$SO_3^{2-} + LOOH \rightarrow SO_3^{\bullet -} + LO^{\bullet} + {}^{-}OH \ (L = \text{lipid}).$$

Alternatively, sulfite may react with lipid radicals for sulfite radical formation. Propofol, as an antioxidant,[26] can donate an electron to quench these and other lipid radicals formed, and in doing so, the propofol radicals created can dimerize to propofol dimer (fig. 5). The formation of propofol dimer quinone can result from a similar oxidation of propofol dimer (fig. 6). Propofol may be particularly effective in quenching lipid radicals because of its high miscibility with lipid. The effect of ascorbate to inhibit these processes is likely due its antioxidant effects.[27] Its high effectiveness in inhibiting propofol dimerization may relate to its high water solubility that may allow a direct quenching of the aqueous sulfite radical.

Of interest is that added ferrous iron, or lipoxidase, caused propofol dimerization in propofol emulsions containing no sulfite. The effect of ferrous iron to facilitate propofol dimerization may be due to the property of iron, as a transition metal, to catalyze lipid peroxidation in the presence of oxygen. Second, ferrous iron can convert lipid peroxides to lipid oxyl and peroxyl radicals.[28] Both can cause the formation of lipid radicals that in the process of being quenched by propofol would generate propofol radicals. The ability of lipoxidase to cause propofol dimerization in lipid may relate to the fact that lipoxidase catalyzes lipid peroxidation. Furthermore, it is an iron-containing enzyme capable of electron donation.[29]

The inability of desferrioxamine in our reaction mixtures to inhibit and in fact increase propofol dimerization in the presence of sulfite and peroxidized soybean oil suggests that the role of trace iron is complex. Trace metals are known to react with sulfite[19] and are presumed to be present in all our reaction mixtures, including the propofol emulsions. Trace metals may therefore be involved in the reactions studied. Stimulatory effects of iron-chelated desferrioxamine (ferrioxamine) on some redox activities has been reported previously.[30]

The *in vivo* effects of propofol dimer quinone and its semiquinone, propofol dimer, have not been studied. Quinones are involved in a number of toxicity-related reactions, including redox cycling to form reactive oxygen species, adduction with DNA, glutathione depletion, and lipid peroxidation.[13] This study shows a role of lipid in sulfite-dependent propofol dimer and dimer quinone formation and implicates lipid peroxides as contributing factors to this reaction in emulsions. Propofol dimerization in sulfite-containing propofol emulsions during longer-term air exposure may be minimized by reducing emulsion unsaturated lipid content or, more directly, by the addition of small quantities of vitamin C.

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