Stability of Vitamin A in Oil-In-Water-In-Oil-Type Multiple Emulsions

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ABSTRACT: The stability of vitamin A was studied in thee different emulsions: oil-in-water (O/W), water-in-oil (W/O), and oil-in-water-in-oil (O/W/O). The stability of retinol (vitamin A alcohol) in the O/W/O emulsion was the highest among the thee types of emulsions; remaining percentages at 50°C after 4 wk in the O/W/O, W/O, and O/W emulsions were 56.9, 45.7, and 32.3, respectively. With increasing peroxide value of O/W and W/O emulsifiers, the remaining percentage of vitamin A palmitate and retinol in the emulsions decreased significantly, indicating that peroxides in the formulae accelerate the decomposition of vitamin A. Organophilic clay mineral (an oil gelling agent and a W/O emulsifier) also affected the stability of retinol; synthesized saponite was better than naturally occurring bentonite for retinol stability. The stability of retinol in the O/W/O emulsion increased with increasing inner oil phase ratio (ϕ_i), whereas in O/W it was unaffected by ϕ_i. Encapsulation percent of retinol in the O/W/O emulsion, the ratio of retinol in the inner oil phase to the total amount in the emulsion, increased with increasing ϕ_i. The remaining percent of retinol in the O/W/O emulsion was in excellent agreement with encapsulation percent, suggesting that retinol in the inner oil phase is more stable than that in the outer oil phase. Addition of antioxidants (*tert*-butylhydroxytoluene, sodium ascorbate, and EDTA) to the O/W/O emulsion improved the stability of retinol up to 77.1% at 50°C after 4 wk. We conclude that the O/W/O emulsion is a useful formula to stabilize vitamin A.

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KEY WORDS: O/W/O multiple emulsion, retinol, stability, vitamin A.

Vitamin A is essential for animal growth, the optical transduction system, immune system, and differentiation of epithelial tissue (1). For cosmetics and pharmaceutics, vitamin A has been used widely because it is a valuable factor in the control of keratinization in normal skin (2). However, vitamin A, particularly all-*trans* retinol, is sensitive to oxygen, heat, and light and is vulnerable to decomposition in cosmetic formulae, resulting in the loss of vitamin concentration and/or the formation of unfavorable odor. Many studies have attempted to analyze the degradation process (3–5), and many efforts, including formation of collagen microcapsules (6) and addition of antioxidants (7–9), have been carried out to stabilize vitamin A. Tsunoda and Takabayashi (10) studied the stability of all-*trans* retinol in O/W emulsion creams. They found that isomerization from all-*trans* retinol to the 13-*cis* form is accelerated with increasing oil content of the emulsion, and that dehydration to anhydrovitamin A is favored with increasing water content. Semenzato *et al.* (11) investigated the relationship between the physical stability of O/W emulsions and chemical stability of vitamin A palmitate, and they concluded that the stability of vitamin A palmitate strictly depends on the stability of the formulation.

Multiple emulsions, both of oil-in-water-in-oil (O/W/O) type and water-in-oil-in-water (W/O/W) type, have attracted considerable interest because of their potential applications for cosmetic, pharmaceutic, agricultural, and industrial chemicals. Prolonged drug release (12–14) and enzyme immobilization (15,16) have been reported in such multiple emulsions, and drug-releasing behavior and enzyme stability depend strongly upon the stability of emulsions (17–20). Despite their advantage, commercial applications of multiple emulsions are limited. Among the reasons for this are their inherent thermodynamic instability and unexpected fast release of encapsulated drugs. A number of emulsifiers have been studied to improve the stability of multiple emulsions and drug-releasing behavior. For example, a mixture of bovine serum albumin and Span 80 (sorbitan monooleate) was employed for W/O/W emulsions in which sulfacetamide was dissolved in the inner water phase (21). Hameyer and Jenni (22) suggested that a stable multiple emulsion requires a firm interfacial liquid-crystalline film. However, the majority of studies about multiple emulsions is confined to the W/O/W type. Even though O/W/O emulsions are expected to be used for controlled release and stabilization of lipophilic drugs, research on them is limited because of the practical difficulty in preparing a stable outer W/O emulsion.

In previous work, we investigated stable formulation of O/W/O-type multiple emulsions by means of organophilic clay minerals as the W/O emulsifiers (23). Because O/W/O emulsions are composed of thee multiple layers in which inner oil droplets are surrounded by dispersed water and an outer continuous oil phase, oil-soluble compounds can be encapsulated in the inner oil phase. In this work, we propose to use O/W/O emulsion for stabilizing vitamin A.

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MATERIALS AND METHODS

Materials. All-*trans* retinol (150 million IU/g, Kurare Chemical, Osaka, Japan); vitamin A palmitate (1.7 million IU/g, a gift from Nihon Roche, Tokyo); polyoxyethylene hydrogenated castor oil [60 mol of ethyleneoxide (EO) units per molecule, HLB = 14, Nikkol HCO-60, Nikko Chemicals, Tokyo]; organophilic montmorillonite clay mineral (Benton 38, National Lead, Hightstown, NJ); organophilic saponite clay mineral (Smecton DS100, Taiyo Chemicals, Tokyo); polyoxyethylene diisostearate and polyoxyethylene dioleate (14 mol of EO units per molecule, HLB = 7.6, Emalex 600 di-IS and Emalex 600 di-O, Nihon Emulsion, Tokyo); and all other reagents and chemicals were of the highest purity or HPLC grade.

Preparation of O/W/O emulsions. O/W/O multiple emulsions were prepared by a two-step emulsification method as reported previously (23). In the first step, we prepared O/W emulsions; an aliquot of liquid paraffin, in which vitamin A was dissolved, was emulsified in 1,3-butanediol with HCO-60, and then water was added. In the second step, the O/W emulsion was emulsified in oil gel that was composed of organophilic clay mineral (Benton 38 or Smecton DS100), lipophilic nonionic surfactants (Emalex 600 di-IS or 600 di-O), and liquid paraffin. A typical photomicrograph of the O/W/O emulsion is shown in Figure 1.

Peroxide value (POV). POV of surfactants was measured by iodometry as described by Hara *et al.* (24) with a potentiometric automatic titrator AT-118 (Kyoto Electronics, Kyoto, Japan). Various degrees of oxidized HCO-60 were prepared by heating at 60°C for 5–48 h. These surfactants were stored at 4°C prior to use.

HPLC analysis. An HPLC system, Nanospace (Shiseido, Tokyo), equipped with a diode-array detector, SPD-M10AV (Shimadzu, Kyoto), was used to analyze vitamin A. Chromatographic conditions for retinol were as follows: a reverse-phase HPLC column, Vydac 201 TP 104-C18, 4.6 × 250 mm (The Separations Group, Hesperia, CA); 10 mmol/L potassium phosphate monobasic and 2 mmol/L potassium phosphate dibasic solution/acetonitrile (4:6) as a mobile phase; flow rate, 1.0 mL/min; detection at 325 nm; column oven temperature at 40°C. Chromatographic conditions for vitamin A palmitate were as follows: a reverse-phase HPLC column, Capcellpak UG120 C18, 4.6 × 150 mm (Shiseido); methanol as a mobile phase; flow rate, 2.0 mL/min; detection at 325 nm; column oven temperature at 40°C. All analyses were repeated twice, and the average values were given.

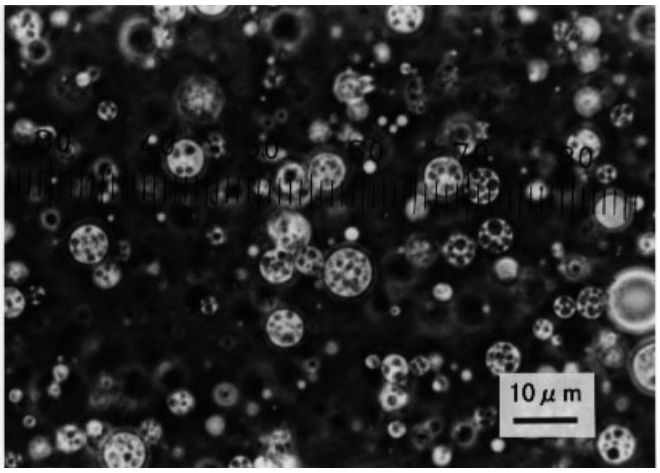

FIG. 1. Photomicrograph of an O/W/O multiple emulsion with organophilic clay mineral. The formula is shown in Table 1.

Encapsulation percentage. Encapsulation percentage, the ratio of vitamin A in the inner oil phase to the total amount in O/W/O emulsion, was measured by the method of Kang and Matsumoto (25). O/W/O emulsions with vitamin A were centrifuged at 2,000 × *g* for 60 min. A small aliquot of separated outer oil phase was withdrawn and analyzed by HPLC. Encapsulation percent was calculated by the following equation:

$$\text{Encapsulation percent} = \frac{VA_T - VA_O}{VA_T} \times 100 \quad [1]$$

where VA_T is the total amount of vitamin A in the whole emulsion, and VA_O is the amount of vitamin A in the outer oil phase.

TABLE 1
Formulas of Emulsions and Retinol Stability

	Components	LP Solution	O/W	W/O	O/W/O
Inner oil phase	Liquid paraffin	99.9	10	—	10
	Retinol	0.1	0.1	—	0.1
Water phase	1,3-Butanediol	—	5	5	5
	Glycerin	—	5	5	5
	Nikkol HCO-60	—	1	0	1
	Methylparaben	—	0.1	0.1	0.1
	Ion-exchanged water	—	to 100	to 100	to 100
Outer oil phase	Liquid paraffin	—	—	27.6	27.6
	Smecton DS100	—	—	2	2
	Emalex 600 di-IS	—	—	0.4	0.4
	Retinol	—	—	0.1	0
Remaining percent of retinol at 50°C after 4 wk		0	32.3	45.7	59.6

RESULTS AND DISCUSSION

Vitamin A stability in different type emulsions. The stability of retinol in thee different emulsions (O/W, W/O, and O/W/O) was studied. Their formulae and remaining percent of retinol at 50°C after 4 wk are shown in Table 1. A simple solution of retinol in liquid paraffin (LP) was also prepared for a control. Retinol dissolved in LP decomposes completely, whereas it is much more stable in the emulsions; 32.3, 45.7, and 59.6% of retinol were found in the O/W, W/O, and O/W/O emulsions, respectively. Even though the composition of inner and outer oil phase of the O/W/O emulsion is the same as that of O/W and W/O (see Table 1), the remaining percent of retinol in the O/W/O emulsion is the highest among the thee emulsions. Because oxidation is one of the main pathways for the degradation of retinol (10), the concentration of oxygen in aqueous and oil phases affects the stability of retinol. The solubility of oxygen in hydrocarbons, such as liquid paraffin, is about 10 times as high as that in water (Bunsen absorption coefficient at 25°C: 28.1×10^2 in octane, 2.83×10^2 in water). Because retinol in the inner oil phase of O/W/O emulsion is surrounded by a multilayer of water and oil, the invasion of oxygen from the surrounding atmosphere can be suppressed by these layers, resulting in the high stability of retinol.

Because retinol decomposed rapidly in an oil (LP) solution and is present at the outer oil phase of W/O emulsion, W/O emulsion was expected to be unfavorable for retinol stability. However, the stability of retinol in the W/O emulsion is fairly good. It is known that organophilic clay adsorbs amphiphilic compounds in its interlayer (silicate layer) and forms an inclusion compound (26). The adsorption study of retinol on organophilic clay showed that 60% of retinol is adsorbed on the clay in an oil gel that has the same composition as the outer oil phase of the W/O emulsion. Because amphiphilic compounds, such as retinol, are adsorbed on the interlayer (silicate layer) of clay (26,30), retinol is present at the adsorbed phase that can be different from the bulk oil phase, leading to the relatively high stability of retinol in the W/O emulsion. However, the stability of retinol in oil gel decreases with increasing organophilic clay, suggesting that the clay enhances the decomposition of retinol. No convincing explanation for the relatively high stability of retinol in the W/O emulsion was obtained, but poor stability of retinol in oil gel in the absence of aqueous phase implies that retinol is present at the interface between water and oil, and that this condition could be important for the stability of retinol.

Effect of peroxide in O/W emulsifier. The decomposition of vitamin A is accelerated in the presence of peroxide (28,29). We investigated the effect of peroxide in O/W emulsifier on the stability of vitamin A palmitate. Vitamin A palmitate, an analogue of retinol, is less reactive to oxygen than retinol; still, it is important as a skin conditioning agent and a nutritional additive (7,8,11). Figure 2 shows the remaining percent of vitamin A palmitate in O/W emulsions at 50°C after 17 d as a function of POV of HCO-60 (polyoxyethylene hydrogenated castor oil). The remaining percent of vitamin A palmitate decreases linearly with increasing POV, indicating that the decomposition of vitamin A palmitate depends on the concentration of peroxide in the O/W emulsifier.

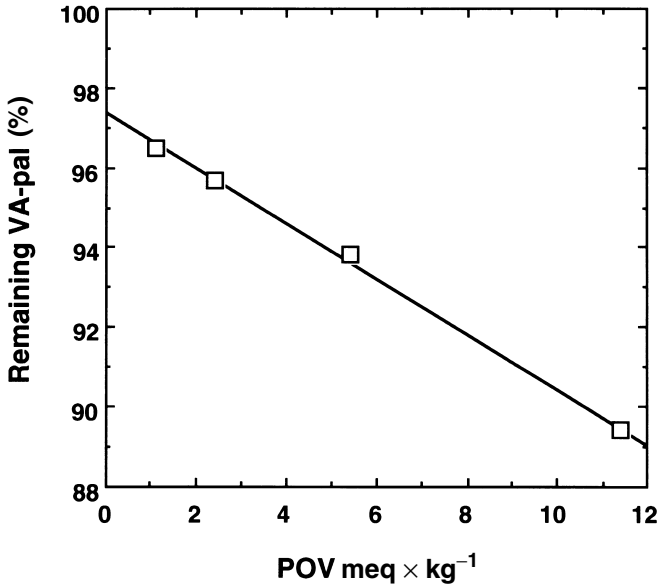

FIG. 2. Effect of peroxide in an O/W emulsifier (Nikkol HCO-60) on the stability of vitamin A palmitate; the remaining percent of vitamin A palmitate at 50°C after 17 d.

Effect of W/O emulsifiers. The effect of W/O emulsifiers on retinol stability in O/W/O emulsions was investigated. Organophilic clay mineral, Benton 38 or Smecton DS100 in this study, is made from aluminum magnesium silicate, and its sodium ion is ion-exchanged with alkyltrimethylammonium salt. This clay mineral adsorbs amphiphilic substances in its interlayer, resulting in the formation of inclusion compounds: such inclusion compounds with organophilic clay and nonionic surfactant work as both W/O emulsifiers and oil gelling agents (30). Benton 38, composed of naturally occurring bentonite, contains significant amounts of metal oxides and salts, e.g., Fe_2O_3, whereas Smecton DS100, prepared from synthesized smectite, contains no such metals. Ferric ion is known as a powerful catalyst for lipid peroxidation though free-radical reactions; the Fenton reaction progresses in the presence of peroxide (31). Emalex 600 di-O (polyoxyethylene dioleate), a nonionic W/O emulsifier that has unsaturated hydrocarbons in its structure, contained a significant amount of peroxide (POV = 324 meq/kg), while Emalex 600 di-IS (polyoxyethylene diisostearate) showed no detectable POV. We prepared O/W/O emulsions by means of these organophilic clays and W/O emulsifiers, and studied the remaining percent of retinol in the emulsions (Fig. 3). The stability of retinol with Smecton DS100 and Emalex 600 di-IS is the highest among four emulsions as expected; 61.8% of retinol was retained at 50°C after 4 wk. On the other hand, retinol with Benton 38 and Emalex 600 di-O degrades most rapidly; only 14.4% was found under the same conditions. The authors propose that the stability of retinol in O/W/O

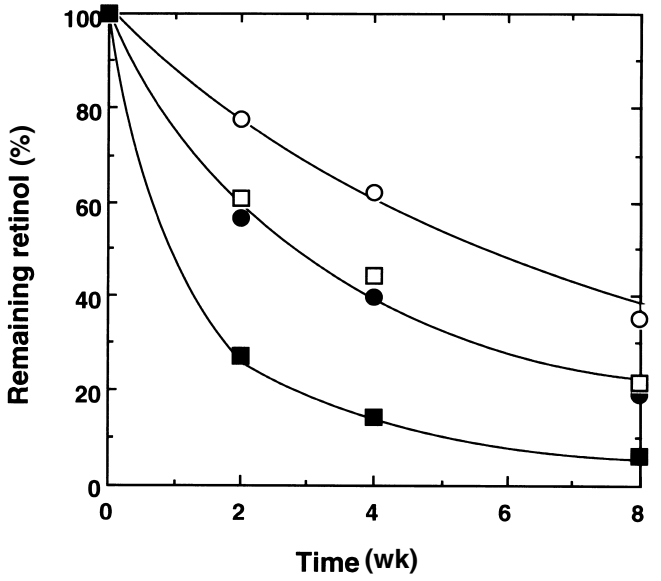

FIG. 3. Effect of organophilic clay minerals and W/O emulsifiers on retinol stability; the remaining percent of retinol at 50°C, with (○) Smecton DS100 and Emalex 600 di-IS, (●) Smecton DS100 and Emalex 600 di-O, (□) Benton 38 and Emalex 600 di-IS, (■) Benton 38 and Emalex 600 di-O.

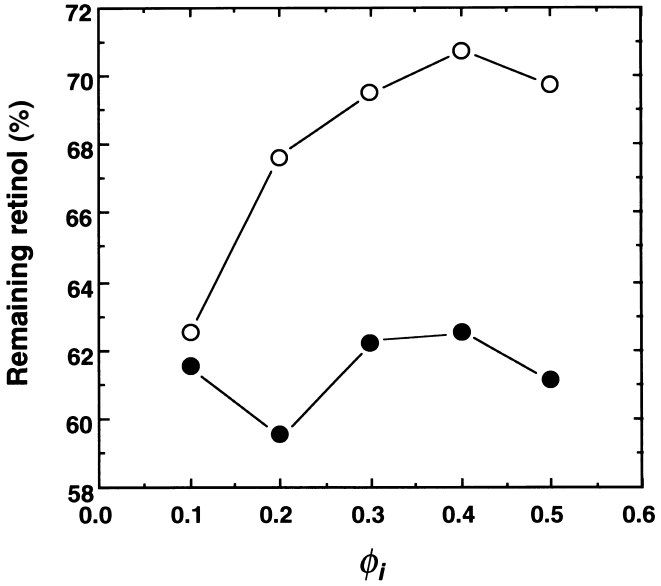

FIG. 4. Effect of inner oil phase ratio (ϕ_i) on the stability of retinol; the remaining percent of retinol at 50°C after 2 wk, (○) in O/W/O, and (●) in O/W.

emulsion is insensitive to W/O emulsifiers because retinol is separated from the outer oil phase by the aqueous phase. However, W/O emulsifiers do affect the stability of retinol. Furthermore, these results imply that the degradation of retinol is enhanced in the presence of peroxides and metals though a radical chain reaction, and that it is important for preserving retinol to remove these catalytic impurities from W/O emulsifiers, even in O/W/O emulsion.

Effect of inner oil phase ratio (ϕ_i). We prepared O/W and O/W/O emulsions with varying ratios of inner oil phase (ϕ_i), and studied the stability of retinol in these emulsions. The remaining percent of retinol in these emulsions at 50°C after 2 wk is plotted as a function of ϕ_i in Figure 4. The stability of retinol in the O/W emulsions does not depend on ϕ_i, whereas in the O/W/O emulsions it increases with increasing ϕ_i at ϕ_i = 0.1–0.4. For the O/W/O emulsion at ϕ_i =0.5, the stability of retinol appears to be decreased, and a slight oil separation is observed in this emulsion. Because the chemical stability of vitamin A depends on the physical stability of the emulsion, as reported by Semenzato *et al.* (11), the decrease of retinol stability at ϕ_i = 0.5 is due to instability of the emulsion. Encapsulation percentage, the ratio of retinol in the inner oil phase to the total amount in the O/W/O emulsion, was measured, and the result is shown in Figure 5. Encapsulation percent increases with increasing ϕ_i for the 0-d sample, and several possible reasons may account for this observation. For example, when the O/W emulsion is "reemulsified" into the outer oil phase at the second emulsification step to make the O/W/O emulsion, the inner oil droplets should come into contact with the outer oil phase at the interface between dispersed aqueous phase and continuous oil phase. Some of the inner oil droplets may be ruptured, and the inner oil phase is absorbed into the outer oil phase, resulting in a decrease of encapsulation percentage; less than 20% of retinol is found in the outer oil phase (Fig. 5). At a constant concentration of retinol in the total formula, the concentration of retinol in an inner oil droplet increases with decreasing ϕ_i. For example, 0.1 g of retinol is dissolved in 10 g of liquid paraffin at ϕ_i = 0.1, while the same amount of retinol is in 20 g of oil at ϕ_i = 0.2. It is impossible to count how many inner oil droplets are

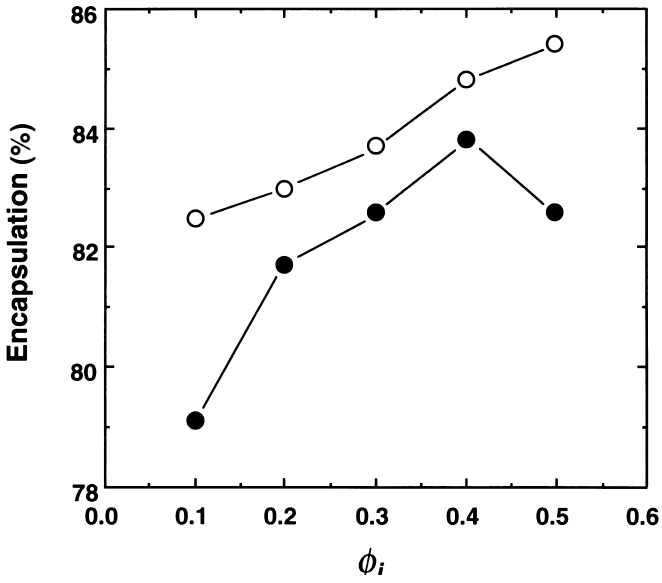

FIG. 5. Effect of inner oil phase ratio (ϕ_i) on encapsulation percentage (the ratio of retinol in the inner oil phase to the total amount in O/W/O emulsion); (○) d 0, (●) 2 wk at 50°C.

ruptured during the second emulsification step, but supposing more or less a constant number of inner oil droplets is broken up in this step, the amount of retinol in the outer oil phase may increase with decreasing ϕ_i. Therefore, the encapsulation percentage increases with increasing ϕ_i at d 0. Alternatively, because the concentration of retinol should attain equilibrium between the inner and outer oil phase, retinol in the inner oil phase will migrate to the outer oil phase, even when the inner oil droplets are intact at the interface of W/O during the second emulsification step. At a constant concentration of retinol in the total emulsion, the concentration of retinol in an inner oil droplet increases with decreasing ϕ_i, leading to a larger difference of retinol concentrations between the inner and outer oil phases. The situation under consideration is represented schematically in Figure 6. The difference of retinol concentrations between the inner and outer oil phases ($\Delta C_{retinol}$) induces retinol to migrate from the inner oil phase to the outer oil phase, and equilibrium will be established. In addition, the initial velocity of migration for retinol increases by increasing the difference of retinol concentrations. Because this difference increases with decreasing ϕ_i, a lower encapsulation percent for lower ϕ_i could be observed.

When the O/W/O emulsions are kept at 50°C for 2 wk, encapsulation percent of retinol appears to decrease 1.0–3.4% (Fig. 5). Although the encapsulation percent at $\phi_i = 0.5$ is the highest among the emulsions at the initial conditions, it decreases substantially after 2 wk. As described above, the physical stability of emulsion at ϕ_i. = 0.5 is unsatisfactory, and the decrease of the encapsulation percent is due to degradation of the emulsion. The decrease of encapsulation percentage at 2 wk becomes larger with decreasing ϕ_i at ϕ_i = 0.1–0.4, and this observation can be explained by the same idea that we have discussed in Figure 6. The difference in retinol concentrations between the inner and outer oil phases ($\Delta C_{retinol}$) causes retinol migration from the inner oil phase to the outer oil phase, and the larger difference of retinol concentrations induces faster migration. Because the difference

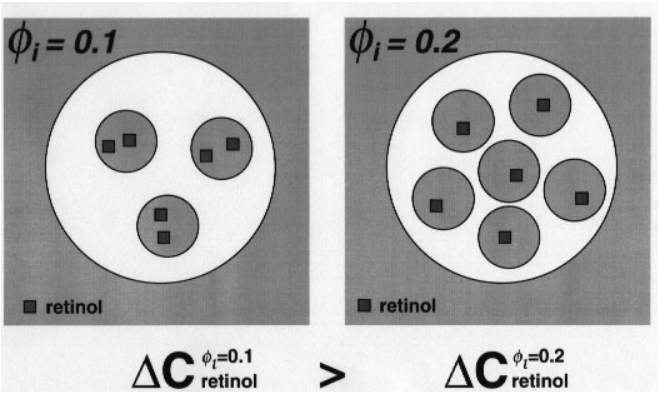

FIG. 6. Schematic depiction of retinol distribution in O/W/O emulsions at the same concentration of retinol; (A) $\phi_i = 0.1$, (B) $\phi_i = 0.2$. The difference of retinol concentrations between the inner and outer oil phase ($\Delta C_{retinol}$) induces retinol to migrate from the inner oil phase to the outer oil phase, resulting in a decrease of encapsulation percentage.

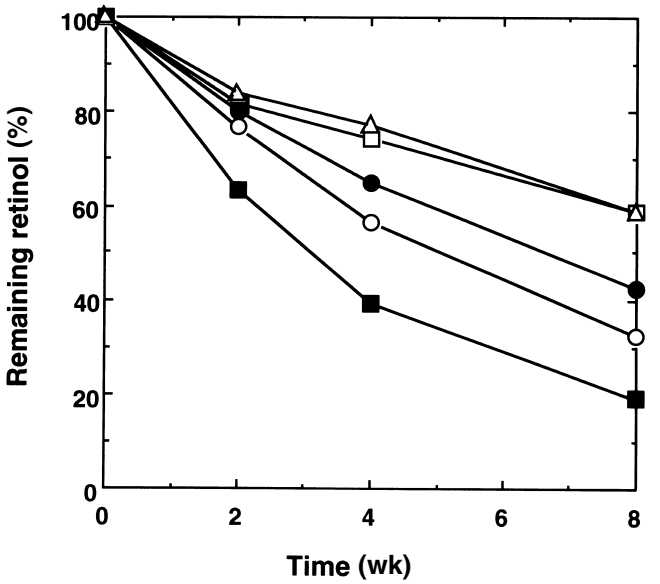

FIG. 7. Effect of antioxidants on retinol stability in O/W/O emulsions; the remaining percent of retinol at 50°C, (○) control, (●) 0.05% BHT, (□) 0.1% sodium ascorbate, (■) 0.1% EDTA, (△) mixture (0.05% BHT, 0.1% sodium ascorbate, 0.1% EDTA).

in retinol concentrations increases with decreasing ϕ_i, the larger decrease of encapsulation percentage is observed at lower ϕ_i.

In Figures 4 and 5, excellent agreement between remaining percent of retinol in the O/W/O emulsions and encapsulation percent after 2 wk was found; the stability of retinol increases with increasing encapsulation percentage, which is consistent with the observation of higher stability of retinol in O/W/O emulsion than in W/O. These results imply that retinol should be kept in the inner oil phase of O/W/O emulsion to stabilize it. It is known that O/W emulsifiers that form micelles in an aqueous phase accelerate the migration of drugs from the inner phase to the outer phase (25). Because organophilic clay mineral consists of a solid-like W/O interfacial membrane in this O/W/O emulsion, the inner oil droplets and micelles that contain retinol are prevented from being absorbed to the outer oil phase, resulting in the high stability of retinol.

Effect of antioxidants. The effect of antioxidants on retinol stability in O/W/O emulsions was investigated with water- and oil-soluble antioxidants, and the results are shown in Figure 7. The addition of 0.05% *tert*-butylhydroxytoluene (BHT), an oil-soluble antioxidant, fairly improves the stability of retinol, whereas 0.1% sodium ascorbate, a water-soluble antioxidant, stabilizes retinol effectively. The remaining percent of retinol decreases by adding 0.1% EDTA, a metal chelating agent, because the addition of EDTA makes the emulsion unstable; we observed a slight oil separation in the emulsion. A mixture of antioxidants (0.05% BHT, 0.1% sodium ascorbate, and 0.1% EDTA) is the most effective to improve the stability of retinol; 77.1% of retinol was retained at 50°C after 4 wk. Because BHT is dissolved in the inner oil

phase, where retinol is held in the O/W/O emulsion, the relative concentration of BHT in the inner oil phase is higher than in the O/W and W/O emulsions that have the same total oil composition. In addition, the inner oil phase of O/W/O is enclosed by the aqueous phase in which sodium ascorbate is dissolved, and it is further surrounded by the outer oil phase.

The results reveal that the O/W/O emulsion formulated with organophilic clay mineral is an effective carrier for stabilizing vitamin A. Furthermore, we expect that this O/W/O emulsion can be useful not only for vitamin A but also for other unstable oil-soluble agents.

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