Stabilization of Retinol through Incorporation into Liposomes

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Chemical and photochemical processes during storage and preparation rapidly degrade retinol, the most active form of vitamin A. Therefore, the efficacy of incorporation into liposomes in order to modulate the kinetics of retinol degradation was investigated. Retinol was readily incorporated into multilamellar liposomes that were prepared from soybean phosphatidylcholine; the extent of the incorporation was $98.14 \pm 0.93\%$ at pH 9.0 at a ratio of 0.01 : 1 (wt : wt) retinol : phospholipid. It was only marginally lower at higher retinol concentrations. The pH of the hydration buffer had a small effect. The incorporation efficiency ranged from $99.25 \pm 0.47\%$ at pH 3 to $97.45 \pm 1.13\%$ at pH 11. The time course of the retinol degradation in the aqueous solution in liposomes was compared to that of free retinol and free retinol with α-tocopherol under a variety of conditions of pH (3, 7, and 11), temperature (4, 25, 37, and 50°C), and light exposure (dark, visible, and UV). The retinol that was incorporated into the liposomes degraded significantly slower than the free retinol or retinol with α-tocopherol at pH 7 and 11. At pH 3, where the free retinol degrades rapidly, the degradation kinetics were similar in liposomes and the presence of α-tocopherol. At pH 7.0 and 4°C in the light, for example, free aqueous retinol was completely degraded within 2 days, while only 20% of the retinol in the liposomes were degraded after 8 days. In general, the protective effect of the liposome incorporation was greater at low temperatures, at neutral and high pH, and in the dark. The results suggest that protection is greater in the solid, gel phase than in the fluid liquid crystalline phase lipids. These results indicate that the incorporation into liposomes can extend the shelf-life of retinol under a variety of conditions of temperature, pH, and ambient light conditions.

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Introduction

Vitamin A is the generic name for a class of nutritionally active, unsaturated hydrocarbons that contain at least one non-oxygenated β-ionone ring with an attached isoprenoid side chain (Willhite *et al.*, 1986). This class (the so-called retinoids) includes retinol, in which the side chain terminates in an alcohol-retinal (terminating in an aldehyde) and retinoic acid (terminating in a carboxylic acid). A number of carotenoids have provitamin A activity, because they can be enzymatically oxidized to a retinoid (Lee *et al.*, 1999a). Retinol that contains all of the *trans* double bonds in the isoprenoid side chain is the most bioactive form of vitamin A. Vitamin A is important in a wide variety of biological functions (Dawson and Okamura, 1990; Sporn *et al.*, 1994). These include embryonic growth and development, vertebrate vision, immune reactions, and epidermal differentiation. It is also a prime candidate for cancer chemoprevention.

All of the members of this group readily undergo degradative reactions that are characteristic of conjugated double-bond systems that result in a decrease, or even a total loss of vitamin activity. These reactions are generally localized in the conjugated isoprenoid side chain and include isomerization to *cis* isomers with reduced activity, molecular fragmentation, and photochemical and chemical oxidation. Such degradation reactions can reduce the available vitamin activity of stored and processed foods by as much as 50% (Sauberlich, 1985). In general, conditions of high moisture, low pH, and high temperature decrease the stability of retinol and its relatives.

Retinol is a fat-soluble compound that only occurs in animal tissue. It is especially abundant in fish and mammalian liver (cod liver oil, for example), milk fat, and egg yolks (Bondi and Sklan, 1984). Due to its hydrophobic character, retinol is usually found in a complex with lipid droplets (milk fat globules) or micelles in foods, where primarily the linear molecule most likely associates with the extended hydrocarbon acyl chains of the lipid components. Such an environment, which is expected to protect retinol from

degradative reactions, can be readily approximated in the lab using a system of multilamellar liposomes that are dispersed in an aqueous buffer. Liposomes are single or multi-layered vesicles that completely enclose an aqueous phase within a phospholipid bilayer membrane. An important aspect of liposomes is the protection that they afford as an encapsulating agent against potentially damaging conditions in the external environment (Kirby et al., 1984; Dziezak, 1988; Kim and Baianu, 1991; Kirby et al., 1991; Park et al., 1994; Reineccius, 1995; Anne and Gregory, 1996; Lee et al., 1999b). Retinol has also been treated as an interesting molecule to be encapsulated in liposomes. The delivery of liposome-incorporated retinol to blood cells has been studied. These systems reduce blood viscosity and cause less lysis of red blood cells than retinol that is not complexed in liposomes (Singh and Das, 1998). Liposomal all-trans retinoic acid has been formulated for treatment of acute promyelocytic leukemia (Douer et al., 2001; Estey et al., 2001). However, the stability of retinol in liposome has not been sufficiently studied.

This study investigated the effect of liposome incorporation on the kinetics of retinol degradation in aqueous buffers. Retinol was incorporated into multilamellar liposomes that were composed of soybean phosphatidylcholine at a wt : wt ratio of 0.01 retinol to 1 phospholipid. The kinetics of the degradation of free retinol, free retinol in the presence of the anti-oxidant α-tocopherol, and retinol that was incorporated into liposomes were measured as a function of pH, temperature, and exposure to ambient or UV light. The results indicated that the incorporation into liposomes slowed the rate of retinol degradation under almost all conditions. In general, the protective effect of incorporation of retinol into liposomes was greater at lower temperatures, at neutral and high pH, and in the dark. The results indicate that incorporation into liposomes may provide a novel encapsulation method for increasing the shelf-stability of foods and nutritional supplements that are fortified with retinol.

Materials and Methods

Materials All trans retinol and L-α-phosphatidylcholine that were isolated from soybeans were purchased from the Sigma Chemical Co. (St. Louis, USA). Vitamin A-acetate that contained α-tocopherol as an antioxidant was purchased from Fluka Chemie AG (Buchs, Switzerland).

Analytical methods Retinol in liposomes was analyzed using a colorimetric assay (Subramanyam and Parrish, 1976). In summary, 0.2 mL of the liposome solution that contained retinol was mixed with 0.6 mL of a chloroform/methanol solvent mixture (2 : 1, v/v). A 0.1 mL aliquot of the organic solvent layer was then transferred to the test tube and 1 mL of a 20 % SbCl$_3$ solution was added. The absorbance at 620 nm was then immediately measured. The retinol concentration was determined by a comparison of the sample absorbance with a standard curve that was prepared using pure retinol.

Preparation of liposomes containing retinol A 1 g quantity of L-α-phosphatidylcholine, 0.01 g of retinol, and 100 mL of a chloroform/methanol solvent mixture (2 : 1, v/v) were put into a 250 mL round-bottomed flask. The solvent was evaporated on a rotary evaporator to deposit a dry lipid film on the flask wall. The flask was removed from the evaporator and 100 mL of a 10 mM glycine buffer (pH 9.0, containing 0.115 M NaCl) and 0.5 g of glass beads were added to assist the lipids hydration. The solution was then mixed on a rotary evaporator (without vacuum) to hydrate the lipids to form multilamellar vesicles (MLVs). The solution was centrifuged 1 h at 80,000 × g, the supernatant removed, and the pellet was washed with 100 mL of a buffer and centrifuged again for 1 h at 80,000 × g. The supernatant was again removed, and the liposome pellet that contained the retinol was diluted with 100 mL of the appropriate buffer (New, 1994).

Stability testing of liposomes during storage One mL of aliquots of the retinol solutions that contained 0.5 mg/mL retinol were placed in glass vials and saturated with oxygen by equilibrating against the atmosphere for 2 h in the dark. Vials for storage in the dark were wrapped in aluminum foil. The other solutions were stored under ambient room fluorescent lights or under UV illumination.

Results

Incorporation of retinol into liposomes The incorporation efficiency of retinol into liposomes of soybean phosphatidylcholine was 98.14 ± 0.93% when retinol was added to lipids at a ratio of 0.01 g retinol to 1 g phospholipid and the mixture was hydrated at pH 9. The incorporation into liposomes decreased slightly and monotonically with increases in the hydration buffer's pH; incorporation efficiency decreased from 99.25 ± 0.47% at pH 3 to 97.45 ± 1.13% at pH 11 (Table 1). The incorporation efficiency at pH 9 decreased only slightly when retinol was added at higher wt. fractions. For example, at a wt. ratio of 0.05 : 1, the incorporation efficiency was 96.17 ± 2.94% (Table 2).

Retinol degradation at neutral pH Retinol solutions at 0.5 mg/mL were prepared in a pH 9.0 phosphate buffer. The solutions contained either free retinol, retinol with α-tocopherol, or retinol that was incorporated in multilamellar

Table 1. Effect of solution pH on the efficiency of incorporation of retinol into liposomes (retinol : PC = 0.01 : 1)

pH	Incorporation Efficiency (%)[a]
3	99.25 ± 0.47
5	98.98 ± 0.49
7	98.58 ± 0.61
9	98.14 ± 0.93
11	97.45 ± 1.13

[a]Mean ± standard deviation of triplicate measurements.

Table 2. Effect of the retinol : PC weight ratio on the efficiency of incorporation of retinol into liposomes at pH 9.

| Ratio[a]	Incorporation Efficiency (%)[b]
1 : 0.01	97.89 ± 0.39
1 : 0.02	97.86 ± 0.65
1 : 0.03	97.68 ± 1.10
1 : 0.04	96.98 ± 1.30
1 : 0.05	96.17 ± 2.94

[a]Weight ratio of lipid to retinol.

[b]Mean ± standard deviation of triplicate measurements.

liposomes that were prepared from soybean phosphatidylcholine. These solutions were stored at three different temperatures (4, 25, and 50°C), either in the dark or exposed to ambient room light (Fig. 1A-C). A fourth study monitored identical solutions stored at 37°C, either in the dark or exposed to UV light (Fig. 1D). The time course of the retinol degradation was monitored for eight days. The results are plotted as % of the remaining retinol versus time.

Incorporation into liposomes slowed the rate of retinol degradation compared to free retinol, or retinol with α-tocopherol, at all of the storage temperatures at pH 7.0. This protective effect of liposome incorporation was most dramatic at lower temperatures. Retinol in liposomes degraded very slowly during storage at 4°C in the dark with over 90% remaining after eight days (Fig. 1A). On the other hand, free retinol was rapidly degraded under the same storage conditions with only about 10% remaining after one day of storage. While the presence of α-tocopherol slowed the initial rate of retinol degradation, after eight days of storage both the free retinol and retinol with α-tocopherol were essentially completely degraded (Fig. 1A). Exposure to ambient light had very little effect on the kinetics of degradation at 4°C for retinol, either free or incorporated into liposomes.

The kinetics of retinol degradation were noticeably faster at 25°C (Fig. 1B). Under these conditions, both the free retinol and retinol with α-tocopherol degraded quickly with essentially no detectable retinol remaining after two days of storage. Again, incorporation into liposomes dramatically slowed the rate of degradation (Fig. 1B) with no detectable degradation after two days of storage in the dark. At 25°C, however, the retinol in liposomes degraded much faster with exposure to ambient light. After eight days, only 14.3% of the retinol that was incorporated into liposomes remained during storage under light, while 51.0% remained during storage in the dark. Similar time courses of retinol degradation were seen under storage at 37°C (Fig. 1D). The effects of exposure to UV light at this temperature, however, were more dramatic due to the well-known photochemical effects of UV exposure on retinol (Anne and Gregory, 1996). Despite this, incorporation into liposomes provides partial protection against UV-induced degradation.

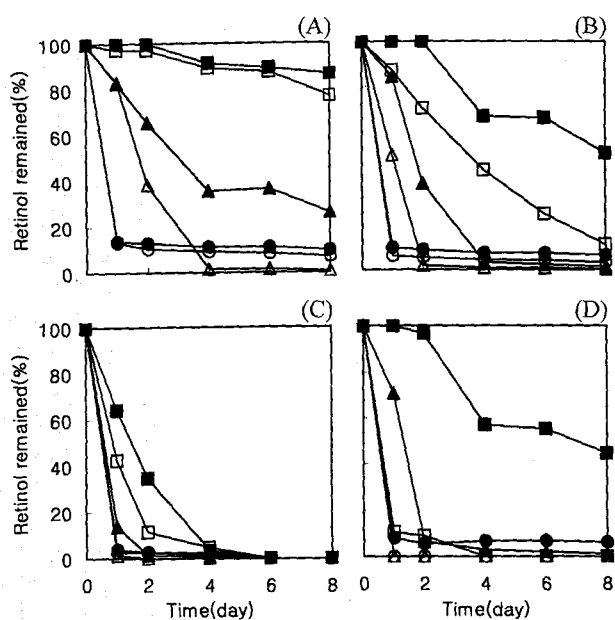The sample that was stored in the dark at room temperature
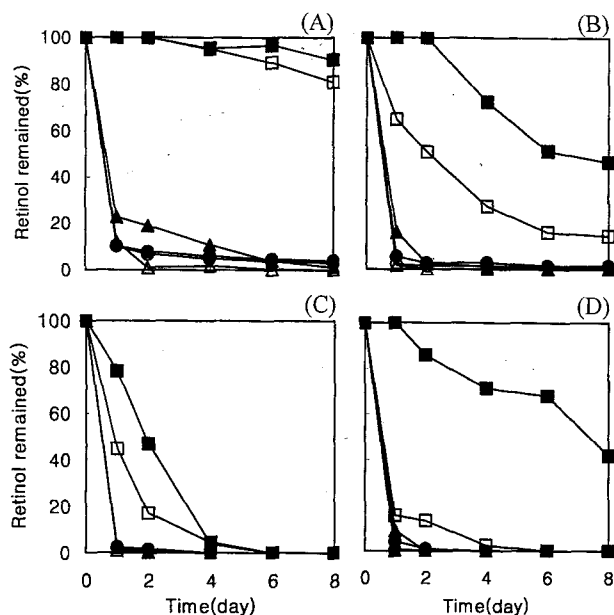
Fig. 1. Stability of retinol in 10 mM phosphate buffer (pH 7.0); the percent retinol remaining plotted as a function of storage time. Buffers that contained free retinol, retinol plus α-tocopherol, and retinol in multilamellar liposomes were stored at (A) 4°C, (B) 25°C, (C) 50°C, and (D) 37°C. Free retinol with light (○) or without light (●), retinol containing α-tocopherol with light (△) or without light (▲), retinol in liposomes with light (□) or without light (■). In (A)-(C), the light was an ambient room light; in (D), the light was an UV source.

displayed a noticeable lag time before the onset of retinol degradation (Fig. 1B). A similar lag time was seen in the time course of retinol degradation in liposomes that were stored at 4°C in either the dark or light (Fig. 1A) and at 37°C in the dark (Fig. 1D). However, liposomes that were stored at 50°C had no lag time under any condition (Fig. 1C).

Incorporation into liposomes also slowed the rate of retinol degradation at higher temperatures (Fig. 1C). After one day of storage, the retinol in solution was completely degraded under all conditions, while only 21.8% of the retinol in liposomes was degraded. Essentially all of the retinol in liposomes, however, was degraded after storage for four days at 50°C under all light conditions.

Retinol degradation at alkaline pH The degradation of free retinol, retinol with α-tocopherol, and retinol incorporated into liposomes was also studied under alkaline conditions in a sodium carbonate buffer at pH 11 at 4, 25, 37, and 50°C. In Figure 2, the results are plotted as % of the remaining retinol versus time. At pH 11 (as at pH 7), incorporation into liposomes slowed the rate of retinol degradation at all of the temperatures that were investigated.

The degradation time course of free retinol at 4°C was quite similar at pH 11 and pH 7, while the degradation of retinol in the presence of α-tocopherol was significantly slower at pH
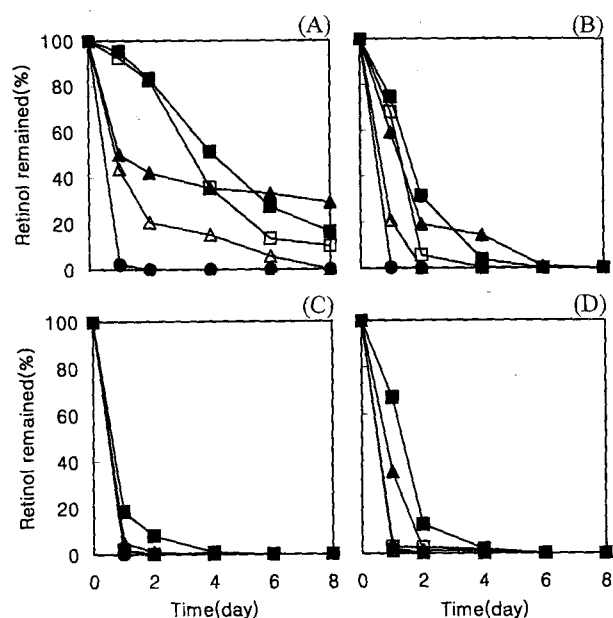
Fig. 2. Stability of retinol in 10 mM sodium carbonate buffer (pH 11); the percent retinol remaining plotted as a function of storage time. Buffers that contained free retinol, retinol plus α-tocopherol and retinol in multilamellar liposomes were stored at (A) 4°C, (B) 25°C, (C) 50°C, and (D) 37°C. Free retinol with light (O) or without light (●), retinol containing α-tocopherol with light (△) or without light (▲), retinol in liposomes with light (□) or without light (■). In (A)-(C), the light was an ambient room light; in (D), the light was a UV source.

Fig. 3. Stability of retinol in 10 mM citrate buffer (pH 3.0); the percent retinol remaining plotted as a function of storage time. Buffers that contained free retinol, retinol plus α-tocopherol, and retinol in multilamellar liposomes were stored at (A) 4°C, (B) 25°C, (C) 50°C, and (D) 37°C. Free retinol with light (O) or without light (●), retinol containing α-tocopherol with light (△) or without light (▲), retinol in liposomes with light (□) or without light (■). In (A)-(C), the light was an ambient room light; in (D), the light was a UV source.

11 than at pH 7 (compare Figs. 1A and 2A). The kinetics of retinol degradation in liposomes were also quite similar at pH 11 and pH 7 with nearly 90% of the retinol remaining after eight days of storage at 4°C at pH 11. Similar results were found at the other storage temperatures. The degradation kinetics for free retinol and for retinol in liposomes were similar at pH 11 and pH 7, while the kinetics of retinol degradation with α-tocopherol were slower at pH 11 than at pH 7. The loss curves for retinol in liposomes also exhibited lag times at pH 11 under the same conditions as found at pH 7 (4, 25, and 37°C). At pH 11, as at pH 7, incorporation into liposomes provided a significant protection against retinol degradation at all of the temperatures that were investigated.

Retinol degradation at acid pH Identical retinol degradation studies were performed in a citrate buffer at pH 3.0. The loss curves for free retinol, retinol with α-tocopherol, and retinol that was incorporated into liposomes that were stored at 4, 25, 37, and 50°C are presented in Fig. 3. In general, at pH 3.0, incorporation into liposomes or addition of the anti-oxidant α-tocopherol had similar effects on the kinetics of retinol degradation.

Free retinol was degraded extremely fast at pH 3. Essentially all of the retinol degraded within one day of

storage at all temperatures. Although the amounts of the remaining retinol after eight days of storage in the dark at 4°C were similar for retinol with α-tocopherol and retinol in liposomes (29.1% and 16.3%, respectively), the degradation curves had quite different shapes (Fig. 3A). Retinol with α-tocopherol exhibited an initial fast phase in which 50.1% of the retinol degraded within one day, followed by a slower phase in which an additional 20.1% was lost over the next seven days. Retinol in liposomes though exhibited an initial slow phase in which 16.3% was lost over two days. This was followed by a faster phase in which an additional 67.0% was lost over the next six days. The initial slow phase in the loss curves for retinol in liposomes was similar to the lag phase that was seen in the loss curves at 4°C at pH 7 (Fig. 1A) and 11 (Fig. 2A). There was no indication of a lag phase in the degradation curves for retinol in liposomes at higher temperatures at pH 3.

The kinetics of degradation of retinol with α-tocopherol and retinol in liposomes were also similar at higher temperatures. Although the presence of α-tocopherol provided slightly more protection against degradation at room temperature (Fig. 3B), incorporation into liposomes provided slightly more protection at 37 and 50°C (Fig. 3C-D). However, the differences in each case were marginal.

Discussion

This study provides evidence that incorporation into multilamellar liposomes significantly protects retinol against chemical degradation under a variety of solution conditions. Under appropriate conditions (neutral or alkaline pH, room temperature or below), the level of protection that is provided by liposomes greatly exceeded that of α-tocopherol, an anti-oxidant that is widely recognized as an effective protective agent (Tesoriere *et al.*, 1997). These data, therefore, provide direct evidence of the efficacy of liposome encapsulation for extending the shelf life of foods and nutritional supplements that are fortified with Vitamin A. It would be useful to have a discussion on the possible mechanism(s) underlying this protection.

Retinol was readily incorporated into liposomes. At 1 wt%, incorporation was 98.1% efficient, and incorporation was greater than 96% for all pH and loading conditions that were examined. Liposomes are self-closed structures that are composed of hydrophobic lipid bilayers and hydrophilic aqueous interior. The hydrophobic molecule could be incorporated in the lipid bilayer. The binding of retinol with liposomes was characterized by fluorescence spectra (Singh and Das, 1998). The exact structure of the retinol complex with the liposomes is still unknown. However, we speculate that retinol binds to lipid bilayers in a manner that is similar to that found for the low molecular weight fluorescent probe diphenyl hexatriene (DPH), a small molecule that contains two aromatic phenyl groups that are attached to each end of a linear conjugated triene double-bond system. Although some ambiguity remains, it is now generally accepted that DPH intercalates into the hydrophobic bilayer in two orientations (Mitchell and Litman, 1998): (1) at the planar interface between the hydrophobic acyl chains and (2) within each leaflet with its long axis parallel to the phospholipid acyl chains. The length of retinol that is calculated from the energy-minimized structures using SYBYL (Tripos, St Louis, USA) is 15.5 Å. The steric energy is 41.64 kcalmol⁻¹. The thickness of the bilayer in multilamellar liposomes is approximately 40 Å (Singh and Das, 1998). We, therefore, expect that retinol can be distributed and stabilized within the hydrophobic core of the liposomes at both the planar interface between lipid leaflets and within each acyl chain region.

Retinol is transported in the plasma as a retinol that is bound to a specific carrier protein, called retinol-binding protein (RBP) (Sporn *et al.*, 1994). RBP, the majority of which circulates in the plasma as a macromolecular complex with transthyretin, delivers retinol to Vitamin-A-requiring cells. However, it has been reported that retinol in liposome transfers spontaneously between liposomes, as well as between liposomes and erythrocytes, with a half life of less than 10 min. This suggests that the transport of retinol, which incorporated into liposomes, may not need the participation of specific transfer proteins in the cell (Fex and Johannesson, 1988).

Retinol, due to the system of conjugated double bonds in the isoprenoid side chain, will react with ambient oxygen. This oxidation is catalyzed by excitation with visible or UV light. Controlling the accessibility of retinol to oxygen is therefore the most likely mechanism by which liposome incorporation slows the rate of retinol degradation. A location for retinol that is deeply buried within the hydrophobic bilayer could therefore protect retinol against degradation by restricting access to molecular oxygen. Although lipid membranes under physiological conditions (in which the lipids are in the fluid liquid crystalline phase) appear to be readily permeable to oxygen (Tesoriere *et al.*, 1997; Subczynski and Hyde, 1998), oxygen permeability is severely reduced in more tightly packed membranes that contain protein (Subczynski and Hyde, 1998). We therefore propose that low oxygen permeability in gel phase lipids is the likely mechanism by which liposome incorporation protects retinol against degradation. A precedent is seen in the effect of acyl chain packing on the permeability of bilayers to water (Lim and Choi, 1991; Huster *et al.*, 1997).

The protective effect of liposome incorporation was greatest at low temperatures and at neutral pH and above. It was lowest at high temperatures and low pH. These results, when rationalized in terms of the effect of temperature and pH on the physical state and structure of the liposome, suggest that solid, gel phase lipids provide the greatest protection against retinol degradation. At 4°C, the multilamellar liposomes that were made from phosphatidylcholine are primarily in the solid gel phase (the transition temperature of soybean phosphatidylcholine is 26.5°C) (Brody, 1982). Oxygen permeability may be lower in the more stable gel phase lipids. This would lead to the slower degradation rates that were seen in this study. Low pH also destabilizes phosphatidylcholine lipids by protonating the phosphate head group. The positively charged head group that is formed destabilizes the bilayer through electrostatic repulsion. Higher oxygen permeability in unstable phosphatidylcholine bilayers at low pH would also lead to the more rapid degradation of retinol that was seen in this study. This hypothesis could be tested by detailed studies of the effect of temperature on retinol degradation in defined lipid bilayer systems with specific transition temperatures.

Conclusion

Retinol was efficiently incorporated into liposomes at pH values that ranged from 3 to 11 and at weight fractions as high as 5%. Incorporation into liposomes significantly protected retinol from chemical degradation. The protective effect was greatest at low (4°C) and intermediate (25°) temperatures, and at neutral and alkaline (pH 11) pH. The effects of temperature and pH are consistent with a mechanism whereby stable gel phase lipids provide the greatest protection against retinol degradation, perhaps by severely reducing the permeability of oxygen. These data indicate that liposome incorporation is an

effective method to improve the shelf-life of foods and food supplements that contain appreciable quantities of retinol, and perhaps, other carotenoids.

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