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Stabilization of liposomes with collagen

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Summary

The potential use of liposomes as drug carriers is still limited by their poor stability under storage conditions and in biological media. As interactions between collagen and lipids have been demonstrated both in vitro and in vivo, we studied the stability of multilamellar liposomes at different temperatures in the presence and absence of type-I collagen solution. The parallel release of 5(6)-carboxyfluorescein from liposomes (due to 'spontaneous' vesicle permeability or induced by detergent) and phospholipid peroxidation were measured as a function of time in the presence of different concentrations of collagen and of two other proteins: albumin and γ-globulin. In the presence of collagen, dose-dependent decreases in both 'spontaneous' liposome permeability and lipid peroxidation were observed. Albumin and γ-globulin had a similar antioxidant effect, but liposome permeability decreased to a lesser extent in the presence of these proteins than at the same concentration (0.8%) of collagen. Thus, the antioxidant effect of collagen cannot explain entirely its stabilizing potential, and collagen molecules would appear to have an additional, specific stabilizing effect on vesicle permeability. Furthermore, collagen as well as albumin and γ-globulin significantly decreased the deleterious effect of detergent on liposomes, probably through a direct interaction with the detergent. We conclude that dispersion of liposomes in a collagen solution may be a means of improving their chemical stability and decreasing their permeability.

Introduction

Liposomes have been tested for use as drug carriers for more than a decade (Machy and Leserman, 1987; Roerdink and Kroon, 1989). They fulfill many of the requirements of a drug delivery system, being composed of biodegradable lipids, relatively nontoxic and apparently well tolerated by human subjects (Lopez-Berestein and Fidler, 1989). Some common pharmaceutical requirements, however, are not yet fulfilled: liposomes have a short shelf-life under storage conditions and poor stability in biological media (Bonte and Juliano, 1986). Several methods have been used to limit leakage of drugs from liposomes, including modification of membrane structure using different lipid components or composition (Senior and Gregoriadis, 1984) and storage of liposomes in a freeze-dried state in the presence of cryoprotective agents (Ozer et al., 1988; Harrigan et al., 1990).

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Weiner et al. (1985) used a novel-sustained release drug delivery system in vivo, in which liposomes and a collagen gel were injected together, intramuscularly or subcutaneously. Release of the drug into the circulation was slower from liposomes sequestered in collagen gel than from vesicles injected alone. The liposomes were thus stabilized by the high viscosity of their environment and also probably by specific interactions with the collagen molecules.

It is well established that lipids interact in vivo with collagen fibres (Le Lous et al., 1982; Barnes, 1985). Cohen and Barenholz (1984) found a strong interaction between collagen or the collagen-like tail of acetylcholinesterase and sphingomyelin liposomes in vitro. More recently, Martinez del Pozo et al. (1988, 1989) described interaction of type-I collagen (organized as molecules or fibres) with phospholipid vesicles. Under acidic conditions, monomeric collagen molecules form complexes with phosphatidylcholine liposomes; at neutral pH, collagen fibrils interact with phosphatidylcholine and phosphatidylglycerol vesicles. Consequently, the protein-lipid interaction produces an enthalpic decrease in the phospholipid phase transition.

The aim of the present study was to determine whether the stability of phospholipid vesicles (permeability of liposomes, oxidation of lipids and detergent-induced release of liposome contents) would be modified by their dispersion in a collagen solution (and not a gel matrix). A comparative study was performed with albumin and γ-globulin molecules.

Materials and Methods

Egg-yolk lecithin (phosphate > 90%) was obtained from Merck (Nogent-sur-Marne, France); and Nonidet P40, 5(6)-carboxyfluorescein (CF; approx. 99% pure by high-performance liquid chromatography: Ref. C7153), albumin (containing less than 1% γ-globulin and essentially fatty acid-free, < 0.005%: Ref. A0281), and γ-globulin (99% pure: Ref. G5009) were purchased from Sigma (La Verpillère, France). Purified bovine type-I collagen, with telopeptides removed chemically (Atelocollagen), was a gift from Bioetica (France).

Collagen characterization

Biochemical characterization of Atelocollagen was performed by high-angle X-ray diffraction, amino acid composition, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and rotary shadowing, as described previously (Ricard-Blum et al., 1985).

Preparation and characterization of liposomes

Egg-yolk lecithin was stored in a chloroform-methanol solution at 4°C. An appropriate amount of lipid solution was dried under nitrogen until the solvent was completely removed. The dried film was then suspended in Tris-HCl buffer (10 mM) containing 5 mM CF, pH 7.4. Multilamellar liposomes were formed by mixing the suspension with a magnetic stirrer, followed by homogenization with an Ultra Turrax until a homogeneous, uniform suspension was obtained. The subsequent phospholipid concentration was about 4% (assayed by the method of Marshall-Stewart, 1980).

Free CF was removed by passing 100 μl of the liposome suspension through a column (50 mm × 8 mm) of fine Sephadex G-50; liposomes (lipid concentration, 0.8%) emerged in the void volume whereas free dye was retarded by the gel. In order to determine the optimal experimental conditions, the osmolarity of the Tris-HCl buffer (10 mM, pH 7.4) used as eluant was adjusted with NaCl to 50–2300 mOsm/kg and the percentage of CF released from the purified liposomes was measured immediately after chromatography.

More concentrated purified liposome suspensions (about 4%) were obtained using the mini-column centrifugation technique (Fry et al., 1978).

Liposomes were characterized by electron microscopy using negative staining. Approx. 1 μl of 0.4% liposome solution was applied to a grid covered with a thick formvar film, followed by the addition of 1 μl of 1% phosphotungstic acid solution. After 15 s, the grid was dried and examined under a Hitachi HU 12A electron microscope at the Centre for Electron Microscopy Ap-

plied to Biology and Geology at Claude Bernard University, Lyon.

Leakage experiments

The percentage of CF leaked from liposomes was measured with a Perkin Elmer Spectrofluorimeter 2000 (excitation at 490 nm and emission at 515 nm). Since the fluorescence of CF is quenched during its encapsulation into liposomes (high concentration of dye), after their purification the leakage of CF into the surrounding medium can be monitored continuously and sensitively as an increase in fluorescence due to its dilution in the external media. The fluorescence signal of a diluted liposome dispersion was thus measured at room temperature before (F_i) and after (F_t) the addition of Nonidet P40 at 100 mg per mg lipid, which solubilized the liposomes completely (100% CF release).

CF leakage was calculated from the equation:

$$\%\text{CF released} = (F_i/F_t + F_t \times C) \times 100,$$

where C ($C = 0.04$) is a correction factor introduced by the dilution of F_t after the addition of Nonidet P40.

Liposome permeability Purified liposomes were diluted with the Tris-HCl buffer solution, 150 mM NaCl (300 mOsm/kg) or 500 mM NaCl (1000 mOsm/kg), or in the same buffer (150 mM NaCl) containing purified type-I collagen, albumin or γ-globulin at different concentrations. The final lipid concentration was 0.4 or 0.04%. These solutions were kept at 8, 20 or 37°C, and the release of CF from liposomes was recorded continuously for 1 week.

Detergent-induced release of liposome contents We applied the same protocol to purified liposomes; we diluted them to a final lipid concentration of 0.08% with the buffer solution in the presence or absence of collagen or albumin. The vesicle suspension was then treated at 20°C with different volumes of Nonidet 0.1% in order to obtain surfactant/lipid ratios of 0.1–0.5 mg detergent/mg lipid (Ruiz et al., 1988). Release of CF from liposomes was measured 30 min later.

Lipid peroxidation

Peroxidation studies were performed on the same liposome suspensions using identical experimental conditions of storage, buffer, temperature and lipid concentration. The extent of lipid peroxidation was estimated by the 2-thiobarbituric acid method (Buege and Aust, 1978) and expressed as equivalents of malondialdehyde (MDA) (mg MDA/mg lipid). A standard curve was drawn using tetraethoxypropane, a precursor of MDA.

We verified that CF molecules did not interfere with the thiobarbituric acid-MDA complex.

Results and Discussion

Determination of optimal experimental condition (CF concentration and buffer osmolarity)

The methodological aspects of measuring the stability of liposomes in vitro, using the CF assay, have previously been described by Lelkes (1984). The latter author and others used CF at very high concentrations (100 or 200 mM), corresponding to an osmolarity inside the liposomes of 275 or 550 mOsm/kg. In order to avoid leakage of CF due to osmotic gradients between the interior of the liposomes and the external media, they suspended the vesicles in isoosmotic buffer. Weinstein et al. (1981), however, dispersed purified liposomes prepared with 100 mM CF, in 600 mOsm/l buffer to limit leakage of CF to 25%. We prepared multilamellar liposomes with an average diameter of 300 nm (as measured by electron microscopy, Fig. 1) and in a preliminary study, we determined the lowest possible concentration of CF and the osmolarity of the external buffer that would result in the least leakage of CF from the liposomes.

A curve giving the variation in the percentage of CF quenching as a function of CF concentration (Fig. 2), showed that there was still 97% quenching at 5 mM CF. We therefore prepared liposomes with CF at 5 mM and obtained a total osmolarity (CF + buffer) inside the vesicles of 50 mOsm/kg. We then measured the percentage of CF leakage immediately after chromatography using external buffer as eluant at different osmo-

larities of 50–2300 mOsm/kg. As shown in Fig. 3, complete release of CF was observed at 50 mOsm/kg and an osmolarity of at least 300 mOsm/kg was necessary to reduce the release: 18.4% at 300 mOsm/kg and 6.6% at 2300 mOsm/kg. The time-dependent release of CF from liposomes was then studied in buffers with two different osmolarities of 300 and 2300 mOsm/kg (Fig. 4); as the two graphs were essentially the same, for the rest of the study we used liposomes containing 5 mM CF, purified and incubated in a buffer with an osmolarity of 300 mOsm/kg, which is more compatible with that of human serum.

Effect of collagen on liposome permeability and lipid oxidation

Hernandez-Caselles et al. (1990) showed that both liposome permeability and peroxidation increase with increasing storage temperature. We obtained the same results in the presence or absence of collagen.

In the absence of collagen In the absence of collagen, CF was completely released after 400, 60, and 40 h at storage temperatures of 8, 20 and 37°C, respectively (Fig. 5A). At 8°C an induction period of 100 h was observed, and CF was released slowly (0.14%/h); after 100 h, the rate increased and reached 0.6%/h. At 20 and 37°C,

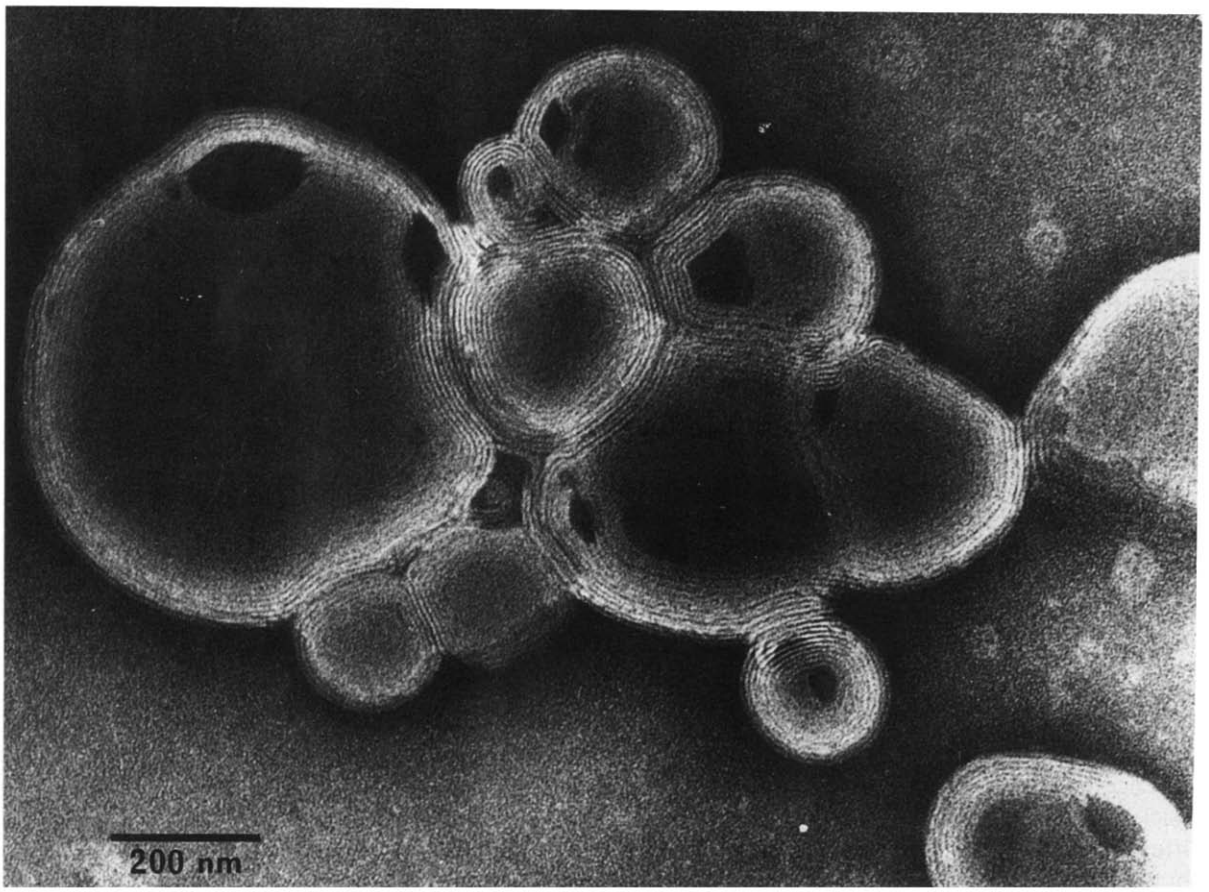


Fig. 1. Transmission electron micrograph of multilamellar liposomes prepared from egg-yolk lecithin and visualized by negative staining.

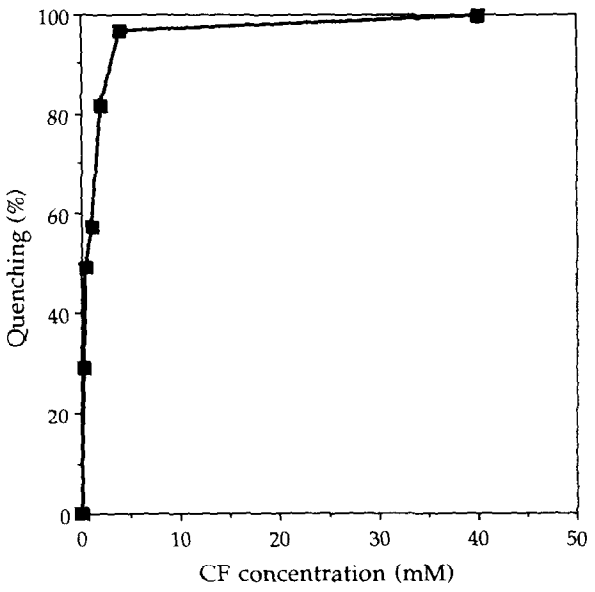


Fig. 2. Fluorescence quenching as a function of 5(6)-carboxyfluorescein (CF) concentration. CF quenching was determined by direct measurement of the fluorescence of different concentrations of CF in solution at pH 7.4 (excitation, 490 nm; emission, 515 nm).

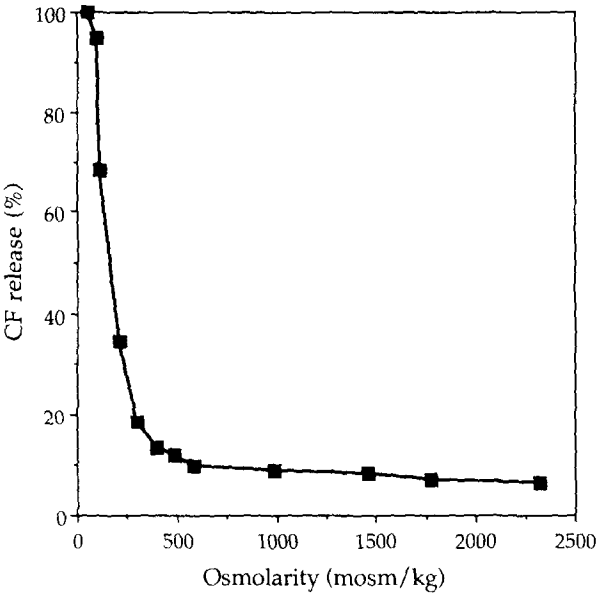


Fig. 3. Osmotic sensitivity of purified multilamellar liposomes prepared with 5 mM 5(6)-carboxyfluorescein (CF). Chromatography columns were equilibrated with buffers at different osmolarities; the percent release of CF from liposomes was determined by measuring pre- and post-detergent fluorescence in vesicle suspensions immediately after their elution on fine Sephadex G-50.

no induction period was observed and CF leakage reached 1.6 and 10%/h, respectively. Parallel measurements of lipid peroxidation (Fig. 5B) showed that 34, 24 and 14 h were necessary to reach a level of lipid peroxidation corresponding to 1×10^{-4} mg MDA/mg lipid at storage temperatures of 8, 20, and 37°C, respectively. No induction period was noted.

These results corroborate those of Hunt and Tsang (1981), who demonstrated a relationship between liposome permeability and lipid peroxidation as measured by the thiobarbituric acid method. Egg-yolk lecithin contains an appreciable amount of unsaturated phospholipids (approx. 35% oleic acid and 20% linoleic acid), which may undergo oxidation. Konings (1984) defined the chemical process of lipid peroxidation as the reaction of an oxidant initiator with an unsaturated fat to form hydroperoxides and cyclic peroxides (leading to the production of MDA measured by the thiobarbituric acid method). Tanfani and Bertoli (1989) showed a linear relationship between liposome permeability and the percent-

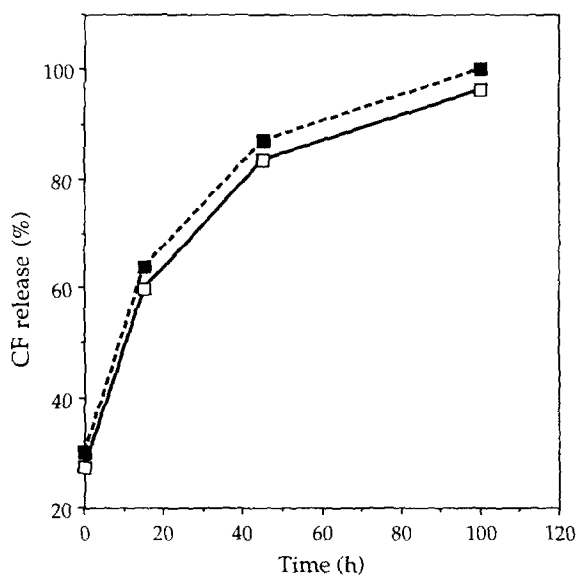


Fig. 4. Effect of buffer osmolarity on time-dependent release of 5(6)-carboxyfluorescein (CF) from liposomes. Lipid concentration, 0.04%. After purification, liposomes were incubated at 20°C in buffer at 300 mOsm/kg (■) or 1000 mOsm/kg (□).

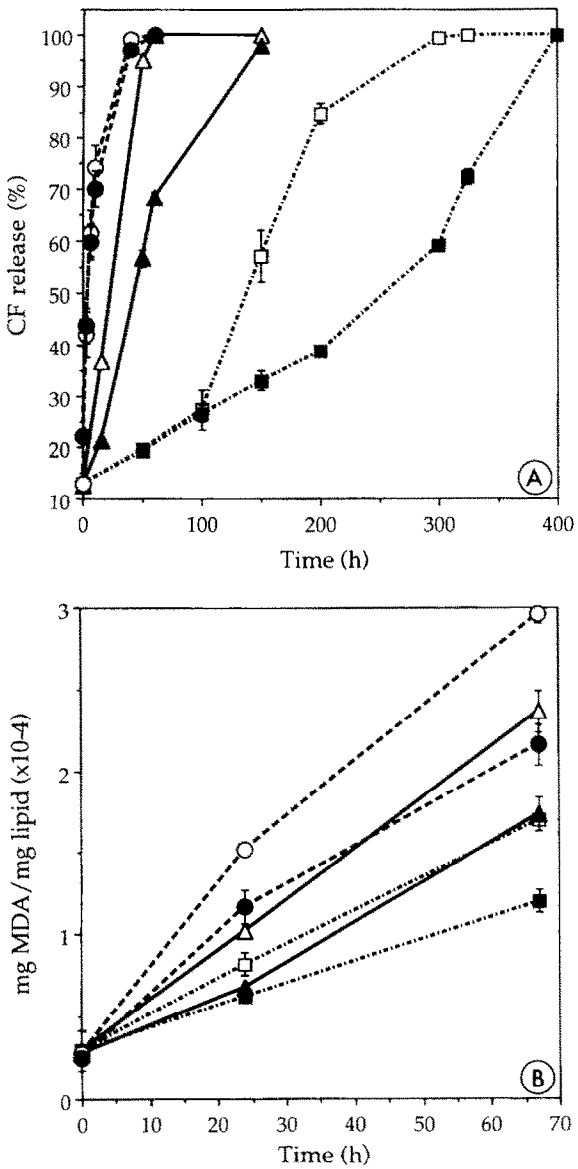


Fig. 5. Temperature dependence of liposome permeability (A) and peroxidation (B) in the presence and absence of 0.8% collagen. Lipid concentration, 0.4%. Each point is the mean (± SE) of three determinations. Control at 8°C (□); control at 20°C (△); control at 37°C (○); collagen at 8°C (■); collagen at 20°C (▲); collagen at 37°C (●); CF, 5(6)-carboxyfluorescein; MDA, malondialdehyde.

age of hydroperoxides incorporated into bilayers. Once formed, hydroperoxides lead to the formation of many degradative products, such as fatty acids (Slater, 1986), which induce fusion between liposomes and leakage of their contents (Crowe et al., 1989). Kunimoto et al. (1981) reported that first the outermost lamellae are damaged by peroxidation and then the damage proceeds sequentially towards the centre of the liposome. They demonstrated that peroxidation did not result in the formation of pores of limited size but disrupted the membrane structure severely. In agreement with their results, we noted that the large increase in permeability observed at 8°C after a certain lag indicates that accumulation of peroxidized products in membranes is probably necessary to cause perturbation of barrier function.

In the presence of collagen In the presence of collagen (0.8%), we observed a stabilization of liposomes, with decrease in both lipid oxidation and permeability compared to the control (Fig. 5). A similar antioxidant effect (about 25% decrease in lipid peroxidation) occurred at the three storage temperatures studied (Fig. 5B), but the decrease in the percentage of CF leakage from liposomes was significant only at 8°C (decrease of 54%) and 20°C (42%) (Fig. 5A). As shown in Fig. 6, collagen had dose-dependent (logarithmic) effects on membrane permeability at concentrations greater than 5×10^{-2}% (Fig. 6A), and on lipid peroxidation at concentrations as low as 10^{-4}% (Fig. 6B). It is worth noting that both permeability and lipid peroxidation depend on the lipid concentration. For instance, in the absence of collagen, CF leakage 70 h after the beginning of the experiments was 85 and 65%, and lipid peroxidation was 7 and 3 mg MDA/mg lipid, for lipid concentrations of 0.04 and 0.4%, respectively. Autooxidation of lipids is accelerated by light (Hunt and Tsang, 1981); therefore, increased opacity due to an increase in liposome concentration could explain the parallel decreases in vesicle oxidation level and liposome permeability. Such differences between the two lipid concentrations were greatly reduced in the presence of a high concentration of collagen: for example, with 0.8% collagen, CF leakage was similar at 0.04 and 0.4% lipid.

Collagen can thus stabilize liposome membranes. The antioxidant effect of collagen

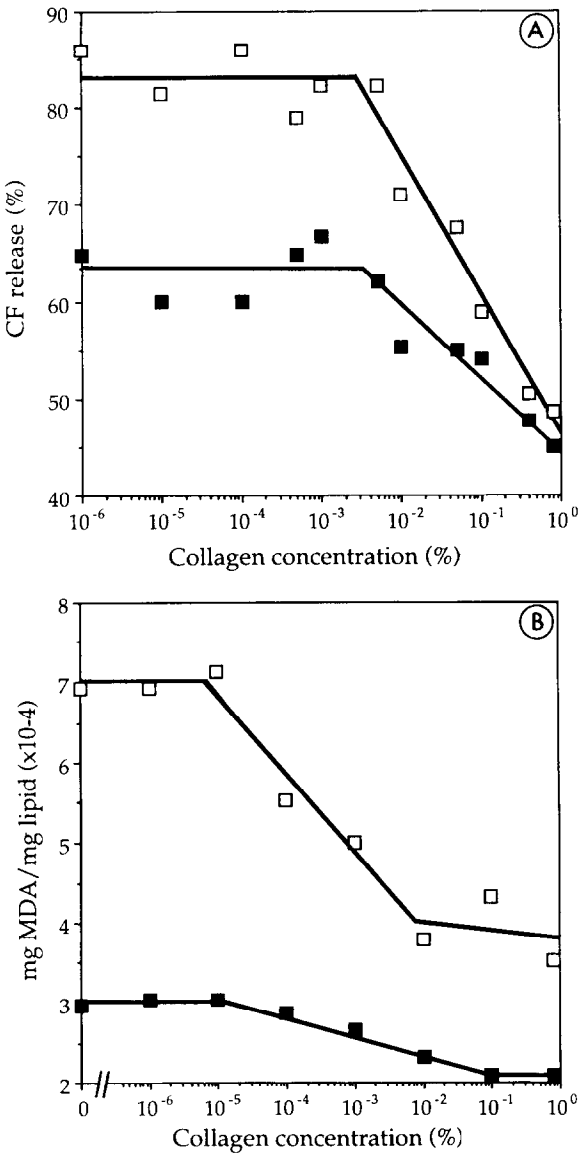


Fig. 6. Rate of release of 5(6)-carboxyfluorescein (CF) (A) and liposome oxidation (B) as a function of collagen concentration. Measurements were made 70 h after the beginning of liposome incubation with collagen solutions at 20°C. Experiments were performed with lipid concentrations of 0.04% (□) and 0.4% (■). MDA, malondialdehyde.

molecules may be associated with its ability to bind to peroxyl radicals and so to inactivate their peroxidation potency. Pokorny et al. (1990) showed that collagen inhibited the oxidation of ethyl linoleate because free peroxy radicals were trapped, principally by the amino groups of collagen. Its antioxidant activity, significant even at very low concentrations (10^{-4}%), is one of the mechanisms by which collagen inhibits the release of CF from liposomes. Other mechanisms are also probably involved in the decrease in liposome permeability, since we observed a dissociation between the antioxidant effect and the decrease in permeability at collagen concentrations greater than 10^{-2}%. At these concentrations, the antioxidant effect reached a plateau, whereas liposome permeability continued to decrease significantly (Fig. 6).

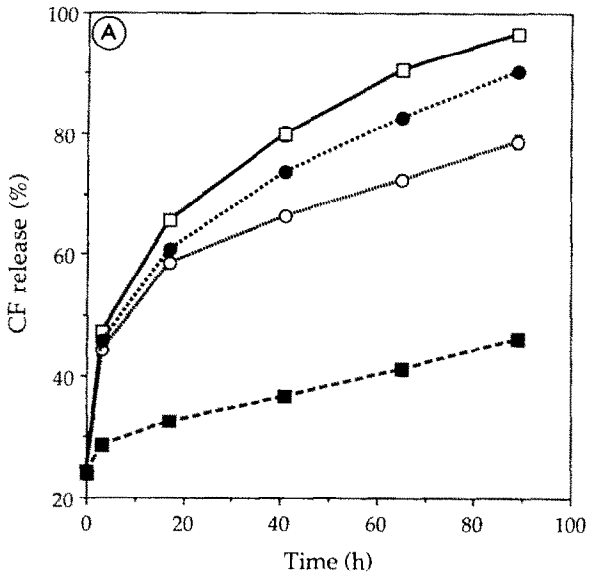
Effect of albumin and γ-globulin on liposome permeability and lipid oxidation

In order to determine whether the effects of collagen molecules are unique, we studied the effects of 0.8% fatty acid-free albumin and 0.8% γ-globulin on liposome stability (peroxidation and permeability, Fig. 7). Using the same experimental conditions, albumin, collagen and γ-globulin had antioxidant effects of 56, 45 and 30%, respectively (Fig. 7B); however, the permeability of liposomes was reduced by only 18% with albumin and 7% with γ-globulin, whereas a 45% decrease was observed with collagen (Fig. 7A).

Antioxidant activity appears to be a general property of proteins that can induce a decrease in liposome permeability, since albumin can also interact with fatty acid and other free peroxy radicals (Goodman, 1958). The much greater effect of collagen on liposome permeability, compared with that of albumin and γ-globulin, must therefore be due to another, specific mechanism. Martinez del Pozo et al. (1988) observed a specific collagen-phospholipid interaction that involves immobilization by collagen of the phospholipid molecules and a consequent decrease in the fluidity of the bilayer. Thus, it is possible that the decrease in liposome permeability observed with collagen is due to a direct interaction with phospholipid. More experiments are necessary to confirm our hypothesis.

Effect of collagen on the stability of liposome in the presence of detergent

In the presence of a detergent, multilamellar liposomes are solubilized by a rapid initial pro-



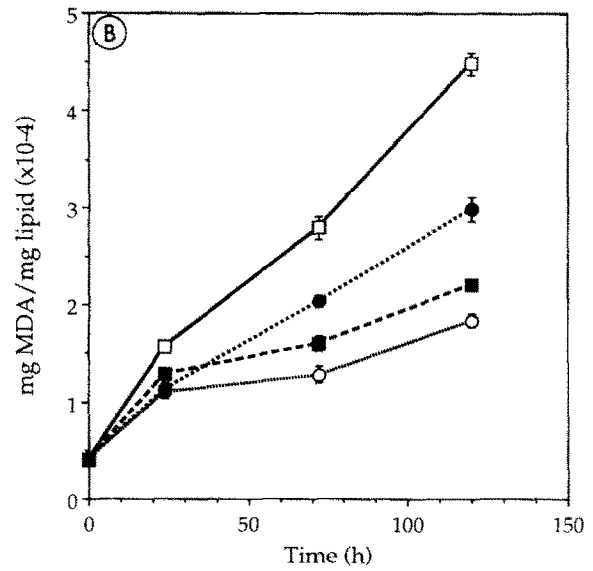
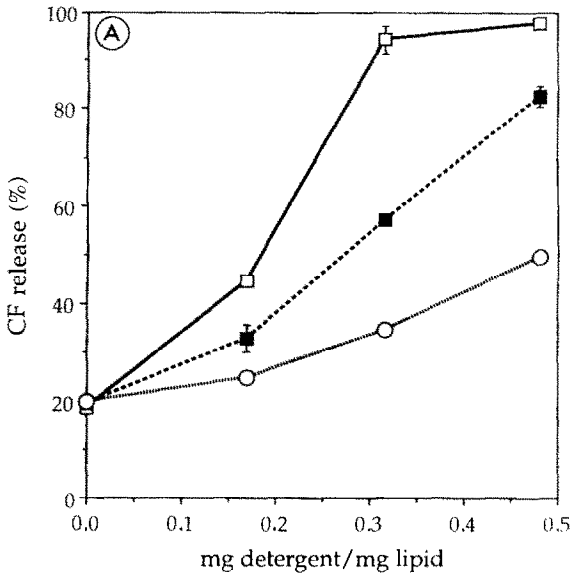


Fig. 7. Liposome permeability (A) and peroxidation (B) with time in the presence of different protein solutions at 20°C. Lipid and protein concentrations were 0.4% and 0.8%, respectively. Each point is the mean (±SE) of three determinations. Control (□); albumin (○); γ-globulin (●); collagen (■); CF, 5(6)-carboxyfluorescein; MDA, malondialdehyde.



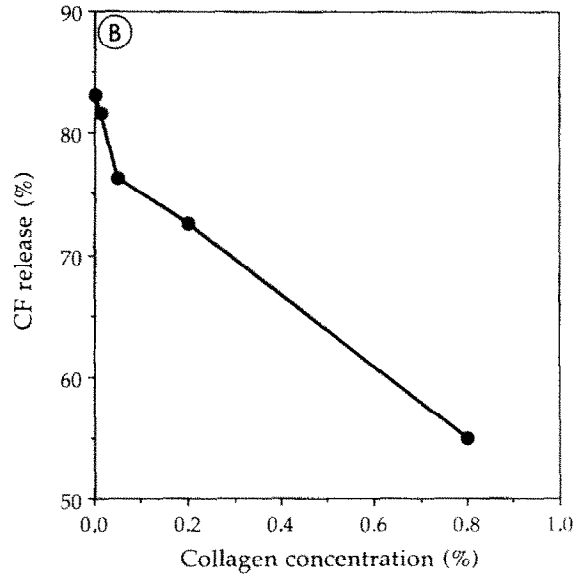


Fig. 8. Effect of detergent (Nonidet P40) on the release of 5(6)-carboxyfluorescein (CF) from liposomes. (A) Percent release of CF from liposomes as a function of Nonidet concentration in the presence and absence of collagen and albumin. Measurements were made after 30 min of incubation of liposomes with detergent at 20°C. Each point is the mean (±SE) of three determinations. Control (□); collagen (■); albumin (○). (B) Effect of collagen concentration on detergent (Nonidet P40)-induced release of CF. Measurements were made in the presence of 0.3 mg detergent/mg lipid after 30 min of incubation of liposomes with detergent.

cess of detergent incorporation and micelle formation, followed by a slow step involving successive peeling off of the individual layers of the liposomes (Muller and Schuster, 1990). Thus,

when we added increasing concentrations of Nonidet to a liposome suspension containing CF, the percentage leakage was increased (Fig. 8A); the release was decreased significantly in the presence of collagen or albumin. Using the method described by Ruiz et al. (1988), we defined R_{50} as the surfactant : phospholipid concentration ratio that would result in release of 50% of the entrapped CF. We determined R_{50} values of 0.18 in the absence of collagen, 0.27 in the presence of collagen and 0.48 in the presence of albumin.

With a fixed Nonidet concentration of 0.3 mg/mg lipid, a dose-dependent decrease in CF release was seen with increasing concentrations of collagen (Fig. 8B).

The protective effect of collagen on detergent-induced CF release is therefore not specific, since albumin behaves similarly. Many authors have described interactions between proteins and detergents (Furth, 1980) and between albumin and detergents (Sukow et al., 1980). The protective effect of collagen and albumin may be explained by the formation of complexes with detergent molecules.

In conclusion, we have demonstrated that collagen molecules decrease liposome permeability by an antioxidant effect and also probably by a specific interaction with phospholipids, the exact nature of which is currently being studied. Furthermore, collagen also provides nonspecific protection against detergent-induced release of CF from liposomes. These results indicate that a liposome-collagen complex could be used either in cosmetology or pharmacology as a drug delivery system.

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